

GENOTYPIC AND PHENOTYPIC DIVERSITY OF CLINICAL AND
ENVIRONMENTAL *VIBRIO VULNIFICUS* STRAINS

By

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GENOTYPIC AND PHENOTYPIC DIVERSITY OF CLINICAL AND
ENVIRONMENTAL *VIBRIO VULNIFICUS* STRAINS

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Vibrio vulnificus is an opportunistic human pathogen causing invasive disease after consumption of raw oysters. Multiple strains are present in shellfish, possessing diverse genetic, phenotypic, and pathogenic characteristics; however, no genotypic or phenotypic markers have been described which reliably predict virulence. In this study, fatty acid methyl ester analysis, biotype, serotype, ribotype (RT) and pulsed-field gel electrophoresis (PFGE) analyses were used in conjunction with mouse LD₅₀ to study the relationship between phenotypic profiles, genetic profiles, and virulence. RT provided the most effective means of clustering *V. vulnificus* and therefore was used in cluster analysis. RT displayed 9 clusters (A-I) at a 50% similarity index. Of clinical isolates, 80% were in clusters B, C, and D; similarly 93% of environmental isolates were in clusters B, C, and D. Cluster B had an equal distribution of clinical and environmental isolates, whereas cluster C contained a very low

proportion of clinical strains. Eighty-eight percent of cluster B isolates were virulent with a mean LD₅₀ of 38 cfu; whereas, only 44% of cluster C isolates were virulent, with a mean LD₅₀ of 2000 cfu. PFGE analyses of 50 blood isolates, from human infections, showed that mortality results from a single strain. Results showed that infection may be initiated by multiple strains, and progresses due to proliferation of high-virulent strains in host blood. In a human case, several oyster isolates matched the PFGE profile of the patient isolate; of all isolates tested, only the patient and matching oyster isolates were lethal in mice. Virulent strains were present in oysters at a concentration of 2×10^3 cfu/gram meat, within a total *V. vulnificus* population of $>10^5$ cfu/gram. Case studies indicated that the mouse iron over-load model may accurately represent the human disease process. Seasonality studies of isolates in oysters showed that springtime shellfish had proportionately higher virulent populations than in summer or fall. Virulent strains did not proportionately increase in oysters subjected to post-harvest elevated temperature.

INTRODUCTION

Consumption of raw oysters from the Gulf of Mexico is responsible for a variety of human illnesses each year. These may be of viral or bacterial origin, and can range from gastroenteritis in healthy persons, to rapidly progressive systemic infections in people with pre-existing hepatic or immune disorders. Among bacterial pathogens isolated from shellfish, *Vibrio vulnificus* is of primary concern to public health officials. Infections are vectored mainly by human consumption of raw shellfish, but may also occur via wound contamination. This invasive organism manifests itself in host tissue as severe primary septicemia, and can cause fulminating lesions in the extremities. Death from septic shock may result in as little as 24 hours (14, 34).

Ecology and Regulatory Standards for *V. vulnificus*

Vibrio vulnificus disease is a problem for shellfish industries, retailers, and regulators. Sickness from this gram-negative organism is a principal public health concern because *V. vulnificus* is natural microflora of estuarine waters (151, 157). Levels of *V. vulnificus* in seawater, sediment, shellfish, and other marine niches are greatly affected by environmental variables, particularly water temperature and salinity. When temperatures are 17 to 30°C and salinity ranges from 5 to 27 parts per thousand (ppt), concentrations of *V. vulnificus* can be as high as 10^3 per mL seawater and 10^6 per gram oyster meat (75,

105, 107, 151). High environmental levels of *V. vulnificus* during spring and summer months correspond with high incidence of reported disease (April to October) (59). In winter months when waters are consistently $<15^{\circ}\text{C}$ or when salinity falls below 5 ppt, *V. vulnificus* levels may be undetectable in the water column and <0.3 cfu/gram in oyster meat. The fate of *V. vulnificus* during cold periods may be due to cell death or to the proposed viable-but-nonculturable state (102); others suggest that bacterial populations "over-winter" in the intestines of marine vertebrates (41).

Considering the seasonal occurrence of *V. vulnificus* and that summertime oysters may contain 10^6 *V. vulnificus*/gram, it is notable that current regulatory standards used to manage oyster harvest do not analyze for the presence of *V. vulnificus*. Instead, regulations rely on measurements of fecal coliforms (e.g., *Escherichia coli*) in over-lying waters to predict health risk. This is a problem because the absence of fecal organisms in oysters do not indicate the absence of *V. vulnificus*. In fact, fecal coliform levels in shellfish may measure 10^2 when *V. vulnificus* are as high as 10^6 , or vice versa (78, 107, 151). Furthermore, post-harvest purification techniques (e.g., depuration) used by the seafood industry to purge oysters of *E. coli*, *Salmonella spp.*, and other non-marine human pathogens, are not as effective in removal of *V. vulnificus*. Data show that *V. vulnificus* may adhere and replicate in oyster tissue when depuration temperature is 23°C or above, and that *V. vulnificus* levels are maintained or reduced only when depurated at 15°C for long time periods (149). Similarly unknown and unmonitored post-harvest temperature abuse may increase levels of *V. vulnificus* in market shellfish (37, 72). Combined, these factors may contribute to increased risk of sickness from shellfish consumption.

When people consume raw oysters, they typically eat a dozen or more shellfish per meal. In doing so, upwards of 100 million *V. vulnificus* cells may be ingested. Consumed populations are comprised of genetically heterogeneous strains, with LD₅₀ values ranging from 10⁶ to 10⁹ cfu (74, 82, 119, 171). Exposure to these populations does not pose a significant threat to healthy individuals; however, a subset of the general public are considered at "high-risk" for *V. vulnificus* disease. High-risk individuals are those with preexisting conditions which compromise host immune response or increase iron concentrations in blood, including: hemochromatosis, cirrhosis, thalassemia, diabetes, cancer, and AIDS (14, 34). Health risks from raw oyster consumption for persons with any of these conditions are 192 times greater than that of healthy individuals (59).

Symptoms associated with *V. vulnificus* disease are chills, hypotension, fulminating lesions of the extremities, edema, and necrotic fasciitis. Infection is acute, progresses rapidly, and may become life threatening in as little as 24 h after ingestion. With a 50% mortality rate, *V. vulnificus* is the most lethal human food borne pathogen reported in the state of Florida (59-61). Since 83% of all *V. vulnificus* cases in Florida are traceable to raw oyster consumption, it is imperative that high risk individuals be educated about hazards of raw shellfish (60). Differences in human susceptibility, as well as variations in bacterial pathogenicity, make public risk difficult to predict.

Virulence Factors of *V. vulnificus*

Determining virulence factors of *V. vulnificus* has been the focus of many studies. Colony morphology, extracellular enzyme production, iron-utilization, and resistance to host immune responses have all been proposed as key indicators of *V. vulnificus* virulence.

However, correlations have not linked any of the above characteristics with disease potential in humans.

Vibrio vulnificus strains are classified by colony morphotype as opaque or translucent (3). Opaque colonies result from cells having thick polysaccharide capsules. These strains show a broad range of virulence in mouse, rabbit, and guinea pig, and increased resistance to *in vitro* phagocytosis by human polymorphonuclear leukocytes (74, 73, 82, 119, 171). Translucent strains have thinner capsular polysaccharide and are considered low-virulence strains. These have LD₅₀ values $\geq 10^9$ per mouse, and because of increased hydrophobicity associated with less capsule, are more susceptible to *in vitro* phagocytosis. Also, serum-induced immune responses are more effective against translucent isolates than opaque strains (9, 82, 129, 161, 171). However, colonies with translucent morphology are considered to be a laboratory phenomenon. Occasionally opaque strains will spontaneously convert to the translucent morphotype, however this switch is rare and usually temporary (168). Because most environmental *V. vulnificus* isolates are opaque, and because of their demonstrated range of virulence, colony morphology should be regarded as a questionable characteristic to use to predict human risk (148).

Many studies have endeavored to link *V. vulnificus* virulence to extracellular enzyme production (e.g., hemolysin, cytotoxin, and cytotoxin) and/or resistance to host defense mechanisms. These factors facilitate entrance into systemic circulation by increasing bacterial penetration of host tissues, and allowing the organism to deter phagocytic elimination. *Vibrio vulnificus* produces a battery of exoenzymes, including

protease, DNase, lipase, esterase, gelatinase, collagenase, and hemolysin (42, 80, 84, 97, 101, 120, 139, 156). Even though these enzymes demonstrate acute cytotoxicity in tissue culture assay, no single enzyme has been conclusively linked to the invasive potential of *V. vulnificus* either by qualitative or quantitative analysis (98, 158, 170). In fact, virulence experiments using strains rendered cytolysin-inactive by transposon mutagenesis showed no decrease in mouse LD₅₀ (167).

Iron-starvation is an initial host response to septic condition. By limiting iron availability to invading organisms, host tissues become innately bacteriostatic, slowing the proliferation of infection (77). During early stages of infection, iron is sequestered by iron-binding proteins (e.g., transferrin, lactoferrin, and haptoglobin) and heme-molecules in host serum; this effectively makes iron unavailable for bacterial growth. However, organisms such as *V. vulnificus*, *V. cholerae*, *Shigella sonnei*, and *E. coli* have developed a means to avoid hypoferric conditions (19, 109, 111). Siderophores are iron-chelating and transport proteins produced by bacteria during times of iron-limitation. Production of hydroxamate and phenolate siderophore proteins enhances bacterial growth (134) in serum by allowing the organism to maintain physiological iron concentrations (hemoconcentration).

Both *in vitro* and *in vivo* studies have demonstrated the marked ability of *V. vulnificus* to sequester iron from host serum (17, 20, 98, 172). Likewise, serum iron levels have marked effects on virulence in mouse models. Following iron-overload with 250 µg of iron dextran per g body weight, LD₅₀ values of virulent *V. vulnificus* strains may decrease by 3.5 log or more (67, 144). Yet, studies comparing siderophore production of

high- and low-virulent *V. vulnificus* strains demonstrate no qualitative or quantitative differences in production of these proteins (134). Although iron sequestering ability has proven importance in *V. vulnificus* infection, iron availability for cell growth is as much dictated by pre-existing host health factors as by bacterial extracellular enzyme production.

Other studies show that *V. vulnificus* is able to resist phagocytosis by mouse peritoneal macrophages and polymorphonuclear leukocytes (82, 152, 153). Bacteriocidal effects of human serum constituents (e.g., opsonins and complement) have demonstrated lesser effects on *V. vulnificus* than other *Vibrio* spp. both *in vivo* and *in vitro* (69, 152, 153).

Phenotypic Bacterial Characterization

Historically, phenotypic criteria have been used to differentiate and speciate bacteria. Differentiation of pathogenic and non-pathogenic bacterial strains is a vital tool in effective public risk assessment and epidemiological investigations for certain human pathogens. Such phenotypic characterizations include biotype, serotype, phage type, multiple antibiotic resistance, inhibitor-resistance, morphotype, fatty acid methyl ester (FAME) composition of cell wall, and outer membrane protein constituents. Phenotypic assays are preferable in most diagnostic laboratories because they are less expensive and laborious; however, results can be variable and subject to lower reproducibility (64). High variability and low reproducibility is most often due to differences in physiological condition of test culture and/or environmental test conditions. Test parameters such as pH, O₂, temperature, and nutrient availability may seriously lessen or alter the expression of some phenotypic characteristics. To obtain adequate and reproducible phenotype results,

care and precision are required. Also, multiple phenotyping techniques are generally used in concordance with genotyping tests to enhance differentiation. Phenotype regimes used in this research are discussed in detail.

Fatty Acid Methyl Ester (FAME)

FAME analysis is a phenotypic characterization used to differentiate and speciate bacteria (81, 96, 100). Fatty acid composition of bacterial cell membranes is highly conserved, but varies from species to species. Over 300 fatty acids and related compounds have been identified using automated fatty acid analysis processes; this makes FAME profiling an excellent taxonomic and comparative tool (127). Profiles consist of a series of peaks representing the presence of individual fatty acids, which are distinguished by the number of carbon molecules within the chain and the degree of saturation. Peaks are obtained by gas-liquid chromatography of cellular extracts, and compared for size and presence. Fatty acids are identified by position within the profile and quantified by determining the area under the peak. These data are analyzed and compared to existing profiles to identify the species or to determine intraspecies similarity (10).

Researchers have used FAME analyses to identify and group virulent species of *Aeromonas* from potable water supplies and diseased individuals in Bangladesh (85). FAME profiles for coagulase-negative staphylococci have proven 92% comparable to more laborious biochemical methods of bacterial identification (85). *Borrelia* spp., *Serpulina hyodysenteriae* and *Leptospira icterohaemorrhagiae* are better speciated by FAME than by serology, as well as *Xanthomonas* spp., *Citrobacter* spp., and *Haemophilus equigenitalis* (70, 87, 100). In epidemiological investigations where pathogenic isolates

of *Francisella tularensis* were misidentified as *Haemophilus spp.* by biochemical assay, FAME and serotype profiles were used together to correctly identify the pathogen (10). Intratypic differentiation of *Citrobacter freundii* has also proven more successful by FAME than other phenotype methods (160).

In experiments to determine the efficiency of an automated FAME profile system for speciation of *V. vulnificus*, 93.7% of 126 isolates tested were correctly identified as *V. vulnificus* (86). FAME has proven utility for speciation, but differences between virulent and non-virulent strains have yet to be conclusively elucidated (9). Further evaluations are necessary to determine correlations between FAME profile and strain virulence.

Biotype

Biotyping strains utilizes a battery of substrates for differentiation. Particularly, carbohydrate fermentation and extracellular enzyme production are commonly used. Traditional biochemical testing is laborious and requires many materials. Numerous plate-tests, tube-tests, and/or chemical reagents are needed to distinguish positive reactions by physical changes in culture medium (e.g., color change, gas production). Commercial kits are available (e.g., API 20E™, Biolog™) to make this task easier, and to test up to 95 different biochemical reactions at once. When more substrates are used in biochemical tests, the test becomes more discriminatory. Biotype analysis is most effective when results are interpreted in conjunction with other phenotype or genotype tests (21, 48, 64, 169). A major problem with biotype procedures is low reproducibility.

All bacterial species can be identified by biotype, and many may be subgrouped by this means. Experiments with enterotoxigenic *E. coli* from sporadic cases of infantile diarrhea have sub-grouped isolates into five principle biotypes (21). Studies examining *E. coli* from intestinal infections of weaned rabbits produced 14 biotypes with 94% of strains from serogroup O130 falling into biotype 14 (28). Pathogenicity of *Yersinia enterocolitica* may be predicted by biotype. Strains testing positive for esculin hydrolysis, salicin fermentation, and pyrazinamidase activity belong to the "non-pathogenic" biotype, while those testing negative are in the "pathogenic" biotype (35). *Vibrio cholerae* O1 may be designated as Eltor or Classical biotype according to their production of hemolysins (116). Other bacteria characterized by biotype include *Staphylococcus aureus*, *Serratia marcescens*, and *Yersinia pestis* (28, 35, 42, 48, 49, 58).

Currently two biotype profiles have been described for *V. vulnificus*: biotype 1 and biotype 2 (159). Biotype 1 includes ubiquitous marine isolates which have biochemical reactivities similar to *V. vulnificus* ATCC 27562. This biotype is identified in clinical laboratories by positive indole production and fermentation of lactose, salicin, and cellobiose (8). Biotype 2 is distinguished from biotype 1 because it is indole-negative, and for the most part restricted to tissues of diseased eels (11, 13). Until recently biotype 2 was not thought to be pathogenic to humans; however, in 1994 several Danish fish-farmers were infected with *V. vulnificus* biotype 2 after handling diseased eels (4). In studies by Oliver et al. (1982) examining Atlantic Coast isolates of *Vibrio* spp., five biotypes were described. Two biotypes from this study contained high percentages of virulent *V. vulnificus* isolates (82.4% and 87.5%); however, only 33.6% of all isolates

tested fell into these clusters. More studies may be necessary to demonstrate a relationship between *V. vulnificus* biotype and virulence.

Serotype

Serological characterization is a widely used technique for bacterial differentiation and speciation. Antigenic variations on cell surfaces are highly conserved and vary greatly between species. Antibodies can be produced to cell wall species-specific cellular epitopes (O-antigens), flagella (H-antigens), or capsule (K-antigens). These antibodies have proven especially useful for rapid bacterial identification, and for certain species (e.g., *E. coli* and *Listeria monocytogenes*), are associated with virulence (21, 71, 130-133, 155, 169).

Serological assays are conducted in several formats including: agglutination, colorimetric, and fluorescent reactions. Slide- and tube-agglutination assays commonly used in laboratories are relatively simple, reliable, and inexpensive. Colorimetric tests, such as enzyme-linked immunosorbant assay (ELISA), use antibodies to bind species- or strain-specific epitopes. Secondary antibodies are conjugated to enzymes which produce color change by substrate cleavage. ELISAs are highly effective and have become the technique of choice for bacterial identification in many research laboratories. When available, fluorescent-antibody tests can be as sensitive as other serological assays, but require the use of epifluorescent microscopes.

Human pathogenic strains of *E. coli* have been grouped by serology. Enterotoxigenic strains of *E. coli* responsible for outbreaks of infantile diarrhea in China have been identified as serotype O126 (169). Virulent strains of *E. coli* which cause diarrhea in pigs belong to serovar O8:KX105, while non-virulent strains are of serotype

O8:K2829 (21). Epidemiological investigations have identified pathogenic serovars of *E. coli* (O6 and O157:H7) and *Yersinia pseudotuberculosis* (1b, 2b, 3, and 4b) in fecal samples of domestic animals, suggesting a link to human disease (33, 47, 165). Pathogenic strains of *Aeromonas hydrophila* and *Aeromonas jandaei* are also differentiated from nonpathogenic strains by serotype (43). Serovars 1 and 6 of *Legionella pneumophila* are known to predominate in human infections, and may be further subtyped by multi-locus enzyme electrophoresis analysis (94). *Vibrio cholerae* strains are traditionally classified as either serovar O1 or non-O1. Although both serogroups can be pathogenic to humans, they tend to exhibit different etiologies (18). Other bacteria successfully grouped by serotype are: *Listeria monocytogenes*, *Salmonella* spp., and *Vibrio mimicus* (1, 107, 115).

Monoclonal and polyclonal antibodies have been produced to H, K, and O antigens of a few *V. vulnificus* strains, but show little correlation between serotype and virulence (88, 130, 150, 155). Biosca et al. (1996) have developed an antibody to LPS which distinguishes *V. vulnificus* of biotype 2 from biotype 1 (11). Antisera produced to a clinical *V. vulnificus* isolate (MO6-24), did not react with environmental strains, and only four of 21 clinical strains were positive by agglutination (57). More research is required to establish relationships between serotype and virulence.

Genotypic Bacterial Characterization

Genotypic characterization techniques are at the forefront of bacterial characterization methods and determination of virulence. A variety of genetic typing methods are available including: restriction fragment length polymorphism (RFLP),

plasmid profile, gene sequencing, and nucleic acid amplification techniques including: polymerase chain reaction (PCR), amplified fragment length polymorphism (AFLP), and randomly amplified polymorphic DNA (RAPD). When compared to phenotype methods, these newer techniques are highly reproducible and less affected by environmental conditions. Consequently, epidemiological researchers have emphasized genotype comparisons to study disease (108). Drawbacks to these methods are expense, labor, and subjective analysis.

Genetic examination by RFLP involves digestion of bacterial DNA using single or multiple restriction endonucleases, electrophoretic separation of DNA fragments, and measurement of genetic profile (108). Profiles are attained by direct-staining techniques, or nucleic acid probes. Direct-staining is commonly used in pulsed-field gel electrophoresis (PFGE), PCR, and RAPD tests, and involves intercalating fluorescent stains (e.g., ethidium bromide). Ribotyping (RT) uses labeled nucleic acid probes, complementary to 16s and 23s gene sequences, to detect DNA restriction profiles with immuno-, radio-, or fluorescent-labeled antibodies. Similarity analysis of RFLP profiles has become easier and less subjective by using computer software. Strains are considered different by the total number of DNA fragments and/or fragment size (64). PFGE and RT have shown promising potential for bacterial speciation and virulence detection, and therefore were used in these studies. Details of each technique are described below.

Pulsed-field Gel Electrophoresis

PFGE analysis applies low-frequency cutting restriction endonucleases to whole chromosomal DNA. These enzymes recognize sequences of six to eight bases in length.

Few cleavages produce DNA fragments larger than 10,000 kilobase pairs (kbp) in length (105). These large fragments are not easily resolved by traditional unidirectional electrophoresis, but PFGE resolves them by alternating electrical currents along different planes at defined time intervals (93, 138). By alternating the direction and duration of electrical current, DNA fragments will reptate through a polymeric gel. Smaller fragments will migrate through gels at a faster rate than larger fragments. A critical advantage of PFGE is that DNA bands with lengths from 2000 to 10,000 kbp are separated with high resolution (6). This makes genetic discrimination of whole DNA more effective than unidirectional electrophoresis (92, 114, 138). Because PFGE profiles are of total chromosomal DNA and not specific gene sequences, the resulting RFLP profiles can have up to 40 fragments (92).

PFGE has been used to determine sources of human disease. Nosocomial infections of *Xanthomonas maltophilia* from five patients were proven to be of different clonal origin by PFGE; two case isolates had identical PFGE profiles compared to those collected from a water faucet and shower pommel (146). Similarly, infectious strains of *Legionella pneumophila* in hospitals have been distinguished from unrelated strains by PFGE (128). In a outbreak of listeriosis in France, PFGE was used not only to trace the vehicle of infection, but to exclude other food sources (66). PFGE has also been used to differentiate strains of *Enterococcus fecium*, *S. aureus*, *E. coli*, *V. cholerae*, *Aeromonas hydrophila*, *Campylobacter hyointestinalis*, *Listeria spp.*, and many others (7, 16, 22-27, 30, 51, 63, 76, 89, 90, 92, 95, 125, 146, 147).

Buchreiser et al. (1995) showed 60 different PFGE profiles among 118 total *V. vulnificus* from three oysters (27). Notably, DNA from approximately 20% of the isolates autodegraded. Nonetheless, this evidence of highly heterogeneous coexisting *V. vulnificus* strains, presumably both pathogenic and non-pathogenic, makes defining hazardous levels of *V. vulnificus* in oysters more difficult than once thought. More testing is necessary to determine if PFGE can be used as an effective means to group virulent *V. vulnificus* isolates.

Ribotype

Bacterial characterization by ribotype (RT) is less discriminating than PFGE, but is very useful for grouping strains. This methodology uses probes specific for 16S and/or 23S rRNA gene sequences (54, 99, 141). Prokaryotic rRNA gene sequences are highly conserved, and have been used to identify strains at the species level (145). Unlike PFGE, RT uses high-frequency restriction endonucleases with shorter recognition sequences (4 to 6 base pairs) which occur in the chromosome. As a result, DNA fragments are smaller, and visually produce smears of DNA through the gel. Smears are transferred to nylon membranes by Southern-blot, and subsequently hybridized with labeled ribosomal gene probes. Profiles are commonly visualized by colorimetric enzyme-linked immunoassay. RT typically produces 10 to 18 fragments per profile.

Studies of *V. cholerae* have shown that all serogroup O12 isolates have identical RT-profiles, while 83% of serogroup O10 belong to a single ribotype (38). Also, *V. cholerae* O139 infections among 21 patients in Bengal were determined to have the same clonal origin by RT profile (45). Isolates of *Pasteurella testudinis* collected from tortoises

with upper respiratory tract disease were distinguished by RT from healthy tortoise isolates (140). *Vibrio anguillarum* O1 isolates, known to possess a virulence plasmid, also have identical RT profiles but differ from those which do not have a virulence plasmid (110). Multi-enzyme ribotype analyses of *Pseudomonas cepacia* isolates from cystic fibrosis patients have demonstrated extreme ribotype variability; this population diversity in diseased individuals has been shown to indicate poor prognosis (123). Studies of *Enterobacter cloacae* showed one RT among community-acquired isolates (164). RT profiles have also been used for typing of *Acinetobacter calcoaceticus*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *E. coli*, and many other species (30, 36, 40, 44-46, 55, 65, 70, 79, 112, 113, 117, 121, 137, 138, 154).

RT was used by Aznar et al. (1993) with *V. vulnificus* isolates and demonstrated a higher degree of heterogeneity among strains of biotype 1 than biotype 2. However, biotype 2 isolates were only distinguishable from biotype 1 when restricted with *Kpn*I. No attempt was made to use RT to differentiate virulence.

Preliminary data from RT studies performed in our laboratory shows that RT may discriminate clinical and environmental *V. vulnificus* isolates. This may lead to a useful way to distinguish strains of high and low virulence. In order to determine the effectiveness of RT as an indicator of *V. vulnificus* virulence, clinical isolates must be analyzed in conjunction with environmental strains. These analyses supplemented with LD₅₀ tests may allow *V. vulnificus* isolates to be grouped by virulence.

Experimental Objectives

The public health threat of *V. vulnificus* has caused regulatory agencies in California, Florida, and Louisiana to mandate that restaurants and retailers place notices on menus, or at points-of-sale that warn customers of the risks of raw shellfish consumption. The Food and Drug Administration has proposed to close the Gulf of Mexico to shellfish harvesting during warm months, because there are no standards for predicting the risk of *V. vulnificus* disease. If methods were available to identify virulent strains of *V. vulnificus*, then more exact regulatory guidelines could be implemented.

Preliminary data indicate that pathogenic strains of *V. vulnificus* appear to show some similar genetic characteristics. However, there is insufficient information to fully establish any genetic relationship with virulence. Further research is needed to determine if single or combinations of phenotypic and genotypic characteristics can be used to predict virulence. With this information, it may be possible to identify virulent populations in oysters. Based on precedents with other bacteria, more research is needed to determine if specific RFLP, FAME, serological and biochemical tests can identify characteristics that are useful for understanding the organism and its disease.

The proposed research will test the hypothesis that genotypic and phenotypic characteristics of *V. vulnificus* may be used individually or collectively to predict pathogenicity and source of *V. vulnificus* isolates. Specific objectives will be to determine:

- 1) the distribution of RT and PFGE profiles among *V. vulnificus* strains
- 2) the distribution of FAME, biotype, and serotype profiles among *V. vulnificus* strains

- 3) correlation among genotypic and phenotypic characteristics
- 4) the distribution of virulent *V. vulnificus* strains in case-associated oysters
- 5) the level of *V. vulnificus* in oysters associated with infection
- 6) whether human mortality results from single or multiple *V. vulnificus* strains in host tissue
- 7) the concentration of *V. vulnificus* in human blood following death
- 8) whether infection is initiated by single or multiple *V. vulnificus* strains
- 9) whether virulent strains show seasonal distribution in the environment, and
- 10) whether post-harvest thermal abuse allows disproportionate proliferation of virulent *V. vulnificus* strains in oyster tissues

MATERIALS AND METHODS

Bacterial Strains

A collection of clinical and environmental *V. vulnificus* strains maintained in the laboratory were cultured in tryptic soy broth (TSB) supplemented with 12%-glycerol and stored in liquid nitrogen (Tables 1 and 2). Environmental isolates include strains previously collected from seawater, sediment, shellfish, and pondwater habitats in Atlantic, Gulf of Mexico, and Pacific waters.

Collection of Oyster Samples and Enumeration of *V. vulnificus* Isolates

Oysters were shucked with sterile knives, weighed, an equal weight of phosphate-buffered saline (PBS) added to each sample, then homogenized for 90 sec in a sterile blender. Twenty grams of oyster homogenate were added to 80 grams of PBS, and further diluted in 10-fold serial increments in PBS.

In the direct-plating technique, 100 μ L of 10^0 , 10^1 and 10^2 dilutions of oyster homogenate were spread-plated in triplicate on tryptic soy agar supplemented to 1% sodium chloride (TSA-1%) and incubated for 4 h at 37°C. Colonies were lifted from TSA-1% plates with sterile nylon transfer membranes (Micron Separations Inc., Westboro, Mass). Colonies were absorbed to membranes for 2 min, then membranes transferred to modified colistin-polymyxin B-cellobiose media (mCPC) (150). Colony blots were incubated for 24 h at 37°C and all cellobiose-fermenting isolates from the lowest countable

TABLE 1: Environmental *Vibrio vulnificus* isolates

Strain MLT#	Source	State	Strain MLT#	Source	State
120	OY	NJ	160	SW	MS
121	OY	NJ	161	SD	MS
122	OY	NJ	162	SD	MS
123	OY	NJ	163	SW	MS
124	OY	NJ	165	SD	MS
125	OY	NJ	166	SD	MS
126	OY	NJ	168	SD	MS
127	OY	NJ	169	SD	MS
128	OY	NJ	170	OY	MS
129	OY	NJ	171	SD	MS
130	OY	NJ	172	SD	MS
131	OY	NJ	173	SW	MS
132	OY	NJ	174	OY	MS
133	OY	NJ	175	OY	MS
134	OY	NJ	176	SD	MS
135	OY	NJ	177	SD	MS
136	OY	NJ	178	OY	MS
137	OY	NJ	179	SD	MS
141	OY	NJ	180	SW	MS
145	OY	NJ	181	SD	MS
148	SD	NJ	182	OY	MS
149	SD	NJ	183	SD	MS
150	SD	NJ	184	OY	MS
151	SD	NJ	185	SD	MS
152	SD	MS	186	OY	MS
153	SD	MS	187	OY	MS
154	SD	MS	188	OY	MS
155	OY	MS	189	SW	MS
156	SD	MS	190	SD	VA
157	SD	MS	191	SD	VA
158	SD	MS	192	SD	VA
159	SD	MS	194	SW	VA

OY = oyster

SD = sediment

C = clam

PW = saltwater pond

SW = seawater

TABLE 1-continued

Strain MLT#	Source	State	Strain MLT#	Source	State
195	OY	VA	276	SW	HI
196	SW	VA	277	SW	HI
197	OY	VA	278	SW	HI
198	SD	VA	279	C	HI
200	SD	VA	280	C	HI
201	SD	VA	281	SW	HI
202	SW	VA	282	C	HI
203	OY	VA	283	C	HI
204	SW	VA	284	C	HI
205	OY	VA	285	C	HI
207	SD	VA	286	C	HI
209	SD	VA	287	C	HI
210	SW	VA	288	C	HI
211	SW	VA	289	C	HI
212	SD	VA	290	C	HI
213	SW	VA	291	C	HI
214	SD	VA	292	C	HI
215	SD	VA	293	SW	HI
217	SD	VA	294	SW	HI
218	SD	VA	295	SW	HI
219	SW	VA	296	SW	HI
220	SD	VA	362	SD	HI
221	SW	VA	363	PW	HI
268	C	HI	364	PW	HI
269	SW	HI	365	SW	HI
270	SW	HI	366	SW	HI
271	SW	HI	367	SW	HI
272	SD	HI	369	SW	HI
273	C	HI	370	SW	HI
274	C	HI	371	SD	HI
275	C	HI	372	SW	HI

OY = oyster

SD = sediment

C = clam

PW = saltwater pond

SW = seawater

TABLE 1--continued

Strain MLT#	Source	State	Strain MLT#	Source	State
385	C	HI	430	OY	FL
399	SW	FL	431	OY	FL
401	SD	FL	436	OY	LA
402	OY	FL	444	OY	LA
403	OY	FL	445	SD	LA
404	OY	FL	447	SD	LA
405	SW	FL	448	OY	LA
406	SW	FL	490	SW	LA
407	SW	FL	491	SW	LA
408	SD	FL	503	OY	MA
409	SD	FL	504	SW	MA
410	SD	FL	527	SD	CT
411	OY	FL	530	OY	CT
412	SW	FL	533	OY	CT
413	SW	FL	1904648	OY	-
423	OY	FL	5112	OY	-
425	SW	FL	Hill	SW	LA
426	SD	FL	938-O	eel	Sweden
427	OY	FL	5112	OY	LA

OY = oyster

SD = sediment

C = clam

PW = saltwater pond

SW = seawater

Table 2: Clinical *Vibrio vulnificus* isolates

Clinical Isolates	Source	Clinical Isolates	Source
1005-T	blood	CVD713-O	blood
1006	blood	CVD713-T	blood
1007	blood	CVD737	blood
1007m	mouse blood	CVD755	blood
1008	stool	E3497	blood
1009	blood	E3939	blood
1010-O	blood	E4125	blood
1010-T	blood	E571	blood
1012	peritoneal fluid	JE2460039	blood
1013	blood	KW694	blood
1014	blood	KW794	blood
1015	wound	LAM624	blood
1355	blood/wound	LCP14001	blood
1365	blood	LL728	wound
1456	blood	MO624-O	blood
1606	blood	MO624-T	blood
1629	blood	SEA10217	blood
1657	wound	UN2814	blood
1674	blood	VvMs	blood

TABLE 2--continued

Clinical Isolates	Source	Clinical Isolates	Source
60-80	blood	1866	blood
230	blood	2098	blood
352	stool	4832	blood
426	wound	5112	blood
427	blood	27562	blood
549	wound	2400112	blood
558	stool	1121PGC	blood
681	blood	398DJ	blood
763	blood	93A-3097	blood
779	blood	93A-3342	blood
825	wound	93A-3953	blood
846	wound	93A-4153	blood
1000	stool	93A-4325	blood
1001	blood	A1402	corneal ulcer
1002	blood	A3490	wound
1003	blood/wound	B308	blood
1004	stool	C7184	blood
1005	blood	C7896	blood

dilutions picked, using sterile wooden sticks, and transferred into 96-well microtiter plates containing 100 μ L alkaline peptone water (APW). Confirmation of isolates as *V. vulnificus* was done by enzyme-linked immunosorbent assay (ELISA), described below.

For the MPN procedure, 1mL of each 10-fold dilution of oyster homogenate was added to 3 tubes of APW and incubated for 24 h at 37°C. After incubation, turbid tubes were streaked to mCPC plates and incubated at 37°C for 24 h. Two *V. vulnificus*-like colonies were picked from each plate, and confirmed by ELISA.

ELISA

Species confirmation was performed by enzyme-linked immunosorbent assay (ELISA) using antibodies specific for intracellular *V. vulnificus* antigens (150). Cellobiose-fermenting colonies were transferred into individual wells of 96-well micro-titer plates each containing 100 μ L APW. Following incubation at 37°C for 3 to 4 h, or until wells were turbid, 25 μ L of each culture was transferred into 96-well ELISA plates containing 25 μ L 0.02% Tween-20 in PBS. To the remaining culture, 75 μ L of 24%-glycerol in APW was added and micro-titer plates stored at -70°C.

ELISA plates were dried in a 37°C incubator overnight to evaporate samples in wells. To reduce non-specific binding of reagents, wells were filled with 200 μ L of 1.0% bovine serum albumin (BSA) in PBS, and incubated at room temperature for 1 h. Albumin was emptied from wells, and plates were firmly slapped onto counter-top covered with absorbent towels to remove residual BSA solution. Monoclonal antibody specific for *V. vulnificus* (Intelligent Monitoring Systems, Gainesville Florida) was diluted 1:4 in PBS, 50 μ L added to each well, then incubated for 1 h at room temperature. Wells were washed

3 times with ELISA wash (17.6 g NaCl, 1.0 mL Tween-20 in 1 L de-ionized water) before treatment with secondary antibody. Goat-anti-mouse antibody conjugated with horseradish peroxidase was diluted 1:200 in PBS, 50 μ L added to each well, and incubated for 1 h at room temperature in the dark. Unbound conjugate was removed from wells by washing 5 times with wash solution. To each well, 100 μ L of freshly prepared substrate solution [10 mg 2,2'-azinno-bis (3-ethyl-benzthiazolin-6-sulfonic acid) in 10 mL 0.05M citric acid, and 30 μ L 30% hydrogen peroxide] was added. Plates were observed for 10 min, positive color changes recorded, and comparisons made with controls.

Fatty Acid Methyl Ester Analysis

In collaborative efforts with our laboratory, FAME profiling was performed by the Natural Energy Laboratory of Hawaii, Kailua-Kona Hawaii. Standard procedures outlined by manufacturer of the Microbial Identification System (MIDI, Inc., Newark Delaware) were followed for fatty acid analysis (86). *Vibrio vulnificus* isolates were grown for 24 h on tryptic soy agar (TSA) with 5% defibrinated sheep's blood at 35°C. Using a sterile plastic inoculating loop, colonies were collected from blood plates and transferred to the bottom of sterile glass tubes. Cells were saponified by addition of 1 mL NaOH in aqueous methanol (45 g NaOH, 150 mL methanol reagent grade in 150 mL de-ionized distilled water) to tubes, vortexing for 5 to 10 sec, and heating to 100°C for 5 min. Lysed cell suspensions were vortexed a second time and reheated to 100°C for 25 min, then cooled to 20°C before methylation.

Fatty acids were methylated using 2 mL hydrochloric acid in methanol (325 mL 6N HCl in 275 mL methanol), vortexed for 5 to 10 sec, heated to 80°C for 10 min, then

cooled. Following methylation, fatty acid methyl esters were removed from the acidic aqueous phase by adding 1.25 mL hexane/methyl-tert butyl ether (200 mL hexane HPLC grade and 200 mL methyl-tert butyl ether) to extracts and then mixing for 10 min. Using a clean Pasteur pipette, the aqueous bottom phase was removed and the top phase washed with 3 mL of dilute NaOH solution (10.8 g in 900 mL de-ionized distilled water). After washing, extracts were centrifuged for 5 min at 2000 rpm and the upper 2/3 transferred into a vial for gas-chromatography (GC) using a 25 m x 0.2 mm phenyl methyl silicone fused silica capillary column. The temperature program ramped from 170°C to 270°C at 5°C per minute, using hydrogen gas as the carrier. Following GC analysis, FAME profiles were analyzed by Sherlock™ Pattern Recognition Software (MIDI, Inc., Newark, DE) and dendrograms produced measuring Euclidean distance. Isolates similar at a Euclidean value of 2, 6, or 10 were identified as the same strain, subspecies, and species respectively (127).

Biotype

Vibrio vulnificus strains of clinical and environmental origin were characterized by utilization of 95 substrates, following manufacturer's instructions (Biolog, Inc., Hayward, Calif.). Cultures were grown on TSA-1% for 24 h at 37°C and suspended in 0.85% saline to an optical density (OD) of 0.28 at 420 nm. Biolog™ plates were inoculated with 150 µL of bacterial suspensions, covered, and incubated at 35°C for 4 h and 24 h. After incubation, plates were observed for color change, and scored as positive or negative. Data were converted to binary code for statistical analysis to determine similarity indexes by binary metric average linkage analysis.

Serotype

In collaborative efforts with Dr. Ron Siebeling at Louisiana State University, standard serological techniques for determining capsule-type (K antigens) and LPS-type (O antigens) were followed. For both serological typing regimes, previously produced monoclonal antibodies against clinical *V. vulnificus* isolates were used (88, 133). In brief, anti-LPS-secreting hybridomas were generated by fusing immune splenocytes collected from female BALB/c mice, which were immunized with whole-cell vaccines, with nonimmunoglobulin-secreting SP2/O-Ag 14 cells (American Type Culture Collection, Rockville, Md.) (88, 133). Similarly, anti-capsule-secreting hybridomas were produced using the same mouse strains and cell lines; however, due to low immunogenicity of pure capsule vaccines, mice were immunized with capsular extracts conjugated with keyhole limpet hemocyanin (133).

For capsule typing, 10 capsular antisera were used to test clinical and environmental isolates of *V. vulnificus*. Bacterial cells were grown on TSA-1% slants and incubated at 37°C for 18 to 24 h. Cultures were centrifuged at 3000 x g for 15 min, resuspended in 10 mL PBS, centrifuged again, and suspended in PBS to produce a cell suspension with an O.D. measuring 0.3 at 405 nanometers. On glass slides, equal volumes (20 µL) of cell suspension and antiserum were mixed by gently tilting slides back and forth for 1 min. Tests were read immediately for agglutination.

For LPS typing, 5 antisera were used to test clinical and environmental isolates of *V. vulnificus*. Cells were grown on TSA-1% slants at 37°C for 18 to 24 hr. Cells were washed from slants using 200 µL of 2% NaCl and 5% glycerol solution, resulting in a

thick cell suspension. Suspensions were transferred to centrifuge tubes (Corning, Inc., Acton, Mass.) and autoclaved for 1 h at 121°C and 16 pounds per square inch. After heat treatment, cells were centrifuged at 4000 x g for 15 min and resuspended in 200 µL of 2% NaCl solution to produce a cell suspension with an O.D. of 0.3 at 405 nm. On glass slides, equal volumes (20 µL) of cell suspensions and antisera were mixed by rocking gently for 1 min and observing for agglutination.

Pulse-field Gel Electrophoresis

The protocol for PFGE was previously described by Buchreiser et al. (27). *Vibrio vulnificus* isolates were grown in Brain Heart Infusion broth (BHI) for 4 h at 37°C. Cultures were pelleted by centrifugation at 4400 x g for 10 min, washed in 10 mL PBS, and repelleted by centrifugation. Bacterial pellets were resuspended in 1 mL of 1X TE buffer (1.58 g Tris-hydrochloric acid and 0.29 g EDTA buffer/L). Equal volumes (200 µL) of cell-suspension and low melting point agarose (Bio-Rad, Melville, N.Y.) (0.11 g low melting point agarose in 9.75 mL 125 mM EDTA) were mixed in microcentrifuge tubes; 100 µL was dispensed into plug molds and allowed to solidify at 20°C. Cell suspensions in plugs were lysed and proteins degraded by treatment with 2 mL deproteinization solution per plug (2 mg proteinase-K/ mL 0.5M EDTA/0.5% N-laurylsarcosine). Plugs were incubated in deproteinization solution at 50°C for 18 h. Following incubation, residual proteinase-K was deactivated by washing plugs 2 times with 4 mL of TE/phenylmethylsulfonylfluoride (PMSF) solution (100 µL of 4 mg PMSF in 100 µL isopropanol/100 mL TE buffer) at 55°C. After deactivation, PMSF was rinsed from plugs

by washing 3 times with TE buffer at 20°C. Unrestricted plugs were stored in TE buffer at 10°C for 1 month.

Cleavage of chromosomal DNA embedded in plugs was carried out according to the manufacturer's recommendations using low frequency cleavage enzymes (e.g., *Sfi*I, *Nof*I, and *Srf*I) (New England Bio-Lab, Beverly, Mass.). Restriction solution consisted of 90 µL sterile water, 15 µL of reaction buffer, 10 µL bovine serum albumin, and 1.0 µL of restriction enzyme. Digestion was performed in microcentrifuge tubes for 16 h at the appropriate temperature for each enzyme.

Sterile plastic loops and glass cover slips were used to transfer plugs into wells of 1% agarose gels (1.8 g PFGE grade agarose, in 180 mL 0.5X Tris-base Boric acid EDTA disodium buffer --TBE) cast in 14 mm x 21 mm gel molds (Bio-Rad). Plugs were sealed into wells with LMP-agarose before electrophoresis. DNA fragments were separated under optimum conditions using a CHEF-DRII system (Bio-Rad). Gels were placed in the CHEF chamber and covered with 2 L of 0.5X TBE maintained at 14 - 18°C by a Lauda chiller unit (Bio-Rad). Buffer was circulated through the chamber at a speed of 1 L per minute using a peristaltic pump (Bio-Rad). Running conditions were 200 volts with the current alternating direction along an orthogonal plane every 1 and 40 sec. Gels were electrophoresed for 24 h and banding profiles visualized by staining with 15 µL of ethidium bromide (0.5 µg/mL) in 300 mL of distilled de-ionized water. Low range molecular markers and lambda concatamers (New England Bio-Lab) were used for reference. Gels were photographed under UV illumination (Fotodyne Inc., Hartland, WI) and scanned into image files for analysis using DNA ProScan software (DNA ProScan,

Nashville, TN). DNA fragment size was converted to binary code for statistical analysis to determine similarity by binary metric average linkage analysis (122).

Ribotyping

RT profiles of *Vibrio vulnificus* strains was done following protocols by Schoonmaker *et al.* (1992) and Sambrook *et al.* (1989), with some modification. Bacterial isolates were grown to late log phase in 15 mL TSB, pelleted by centrifugation at 3000 x g for 15 min, and resuspended in 3 mL TE buffer. To lyse cell suspensions, 250 µL of 10% sodium dodecyl sulfate (SDS), and 50 µL of proteinase-K stock solution (10 mg/mL) were added to pellets, mixed gently, and incubated for 1 h at 37°C. After incubation, 750 µL of 10% hexadecyltrimethylammoniumbromide (CTAB) in 7.5M ammonium acetate solution, pre-warmed to 65°C, was added to each cell suspension, mixed, and incubated for 20 min at 65°C.

After CTAB treatment, DNA was extracted by adding 3 mL of chloroform:isoamyl (24:1) solution to suspensions. Extracts were mixed gently at 20°C for 30 min then centrifuged for 15 min at 1500 x g at 0°C to separate aqueous and organic phases. Following centrifugation, the aqueous phase was carefully removed into a clean centrifuge tube using 1 mL micropipettes with the tip cut to prevent DNA shearing. DNA was precipitated from aqueous solution by adding 3 mL of cold 100% isopropanol and gentle mixing by inverting the tubes. The supernatant was discarded, DNA washed with 5 mL of cold 70% ethanol, and incubated for 10 min at 65°C to evaporate residual alcohol. DNA was dissolved in 4 mL of sterile TE buffer by gentle agitation over night. When completely dissolved, 4 µL of stock RNase solution (1 mg/mL) was added to DNA

solutions and incubated at 37°C for 1 h. DNA was then treated with 80 µL of 3M sodium acetate on ice, followed by 18 mL of cold 100% ethanol and incubation at -20°C for 30 min. After cold incubation, DNA was precipitated by gentle tube inversion and the supernatant discarded. DNA was dissolved in 100 µL TE buffer at 50°C, quantified using a Hoefer UV fluorometer (Hoefer Scientific Instruments, San Francisco, Calif.), and when necessary, diluted with TE to a final concentration of ~100 ng/mL. DNA samples were stored at -20°C until analyzed.

Purified DNA was restricted using a number of high frequency cleavage enzymes (e.g., *HindIII*, *EcoRI*, *BglI*, *SpeI*, and *PstI*), as described by the manufacturer (Gibco BRL, Gaithersburg, Maryland). Banding patterns were compared, and *HindIII* chosen for further ribotyping experiments because of optimum band separation.

DNA restriction solution was prepared in microcentrifuge tubes, and included 10 µL DNA, 7 µL sterile distilled de-ionized water, 2 µL reaction buffer and 1 µL enzyme. Restrictions were incubated for 5 h at 37°C. After digestion was complete, 16 µL of restricted DNA solution was mixed with 4 µL loading buffer (25 mg bromophenol blue, 25 mg xylene cyanole, 4 g sucrose in 10 mL 1X TBE) and loaded into 1% agarose gels. Gels were cast in the electrophoretic sub-cell (Bio-Rad) and covered with 1800 mL of 1X TBE before loading. Electrophoretic conditions of 16 volts for 16 h were chosen. Post-electrophoresis gels were stained using 300 mL de-ionized water and 15 µL ethidium bromide for 30 min, then photographed under UV illumination (Fotodyne Inc.).

DNA was transferred to nylon membranes (Bio-Rad) by the method of Southern (142). Gels were soaked in 100 mL 0.2M HCl for 10 min then treated with 100 mL of

denaturation solution (1.5M NaCl in 0.5M NaOH) for 35 min. Residual denaturation solution was neutralized by treatment with 100 mL neutralizing solution (1.5M NaCl, 0.5M Tris-HCl, and 0.001M Na₂EDTA) for 45 min. DNA was transferred from gels onto nylon membranes via a vacuum filtration system (Pharmacia Biotech, Uppsala, Sweden), using 2X sodium chloride sodium citrate solution (SSC) (0.3M NaCl and 0.03M sodium citrate). Upon transfer, DNA was fixed to nylon membranes using UV-irradiation for 5 min.

DNA fragment banding profiles were hybridized with a mixture of 16S and 23S ribosomal gene probe and visualized using an immunological detection kit (Boehringer Mannheim, Indianapolis, Ind.). Probe was produced using *E. coli* cDNA by avian reverse transcriptase as per the manufacturer's instructions (Boehringer Mannheim). Membranes were placed in hybridization chambers (Techne, Princeton, New Jersey) containing 20 mL standard prehybridization solution (5X SSC, 0.1% n-lauroylsarcosine, 0.02% SDS and 1% blocking agent) and incubated at 42°C for 2 h. Following prehybridization, 6 µL of digoxigenin-labeled probe specific for ribosomal RNA gene sequences was mixed with 10 mL standard prehybridization solution and used to treat membranes for 24 h at 65°C. After hybridization, blots were washed 2 times for 5 min at 20°C, using 20 mL of 2X SSC with 0.1% SDS, then 2 times for 15 min at 65°C in 0.5X SSC with 0.1% SDS.

In preparation for immunological detection, membranes were washed in 20 mL of Genius #1 buffer (10 mM Tris-HCl and 1mM EDTA) for 1 min, then incubated for 30 min in 50 mL Genius #2 buffer (150mM NaCl, 100mM Tris-HCl and 2% blocking reagent). Membranes were treated with 6 µL of anti-DIG-AP antibody conjugate in 30 mL of Genius

#3 buffer (100 mM Tris-HCl, 100mM NaCl adjust to pH 9.5 then add to 50 mM magnesium chloride) for 30 min at 20°C. Unbound antibody-conjugate was removed from membranes by washing 2 times for 15 min with Genius #1 buffer. Bands were visualized by incubating membranes at 20°C in color substrate solution (45 µL NBT-solution, 35 µL X-phosphate solution added to 10 mL Genius 3 buffer) for 16 h. Development reaction was stopped by washing membranes in 50 mL Genius #4 buffer (10 mM Tris-HCl, 1 mM EDTA at pH 8.0). Ribotype profiles were scanned into photo-images and the number of base pairs per band determined using DNA ProScan software (DNA ProScan). Band profiles were converted to binary code and statistically analyzed for similarity indices by binary metric average linkage analysis.

Statistical Tests of Similarity

DNA fragments from all PFGE and RT tests were measured by size using DNA Proscan software (DNA Proscan). Measurements were transferred into binary code, from which similarity was determined. Biotype data were also converted into binary code by positive or negative reaction for similar statistical analysis. These data were plotted by binary metric measurement of average linkage (122) and relationships among strains were examined by cluster analysis. Clusters were arbitrarily defined by percent similarity based upon the over-all number of clusters produced, and the number of isolates per cluster. Data management and analysis were performed with the SAS system (SAS Institute, Cary N.C.) and cluster dendrograms plotted using S-Plus software (Statistical Services, Inc., Seattle, Wash.).

Human Case Studies

Oysters associated with two human infections in the state of Florida during the summer of 1994 were collected from restaurants by state investigators and sent to the laboratory for immediate processing. *Vibrio vulnificus* was enumerated, isolates collected, and subsequently analyzed by PFGE to match shellfish with case isolates. When shellfish isolates were found having identical PFGE profiles to clinical isolates, a representative oyster isolate for each unique PFGE profile was assayed for virulence to determine concentration of virulent strains in oysters.

Collection of Blood Specimen Isolates

Epidemiological information for four *V. vulnificus* cases reported in the state of Florida during the summer of 1994 was kindly provided by personnel from the Florida Department of Health and Rehabilitative Services, and Department of Environmental Protection. Blood specimens were collected from three of the *V. vulnificus* cases. No blood sample was collected from the fourth case; however, a single blood isolate was provided. Specimens were refrigerated at the hospital and shipped overnight to the laboratory in Gainesville, Florida. Upon receipt, sample temperature was recorded, and blood processed immediately.

Blood was serially-diluted in PBS, and 100 μ L plated in triplicate on TSA-1%. After 24 h incubation at 37°C, colonies were enumerated to determine concentration of *V. vulnificus* in patient blood. Approximately 200 colonies per sample were collected and transferred to 96-well micro-titer plates containing 100 μ L 12% glycerine in alkaline

peptone broth, and stored at -70°C. Isolates were confirmed as *V. vulnificus* by ELISA (150). Fifty blood isolates from each case were analyzed by PFGE.

Seasonal Distribution of Virulent *V. vulnificus* Isolates

For seasonal studies, shellfish were collected at monthly intervals from Apalachicola Bay, Florida and transported under refrigeration to the laboratory for immediate analysis. Following enumeration in oysters by MPN, 30 isolates were collected by direct-plating from oysters harvested during spring, summer, and fall, and analyzed for RT. Binary metric, average linkage analysis for similarity was performed on these data as described above, then selected strains tested to determine a possible seasonal distribution of virulence. The chi-squared test of independence (2) was used to test for statistical difference in virulence of seasonal *V. vulnificus* populations.

Effect of Elevated Storage Temperature on Levels of Virulent *V. vulnificus* Strains in Oysters

Oysters used in thermal abuse studies were collected from Cedar Key, Florida, transported on ice to the laboratory, and separated into three groups: 1) samples not to be stored at elevated temperature and processed immediately; 2) samples to be stored at 20°C and 3) those to be stored at 27°C. *Vibrio vulnificus* isolates were enumerated by MPN, and strains for PFGE characterization were collected by direct-plating. A representative sample of isolates, having unique PFGE profiles from each oyster group, were tested for virulence to determine if post-harvest thermal abuse affected the proliferation of virulent strains in oyster tissue. Statistical analysis for differences in proportion of virulent strains, among the three test groups, was determined by chi-squared test of independence.

LD₅₀ Analysis

Vibrio vulnificus isolates were tested for 50% lethal dosage (LD₅₀) using a standard protocol (74). Groups of five two-week old ICR mice (Harlan Sprague, Indianapolis, Indiana) were used per bacterial isolate. Mice received iron-dextran (250 µg iron dextran/gram body weight of mouse) 2 h prior to bacterial challenge. Broth cultures of *V. vulnificus* were grown for 4 h at 37°C in BHI until late log phase. Cultures were pelleted by centrifugation at 4400 x g for 15 minute, resuspended in PBS, and adjusted by optical density to 0.64 at 420 nm, yielding a cell density of ~10⁸ cells/mL. Following subsequent 10-fold dilution of cell suspensions, mice received 0.5 mL intra-peritoneal injection of bacteria containing approximately 10³, 10², 10¹, or 10⁰ cells per mouse. Bacterial suspensions were spread-plated in triplicate TSA-1%, incubated at 37°C for 24 h, and colonies counted to enumerate bacterial concentrations. Mice were observed for a total of 48 h for mortality, and LD₅₀ calculated by the method of Reed and Muench (118). Animal study protocols were approved by the University of Florida Institutional Animal Care and Use Committee protocol #4191.

Virulence assays

Virulence assays were used in seasonal and thermal abuse studies instead of LD₅₀ assays. These tests did not determine a specific lethal dose for the bacterial isolate tested; instead it determined if strains had high or low virulence. Isolates were considered high-virulent if they killed 50% of a mouse population inoculated with ~10³ *V. vulnificus* cells; if 50% of the population was not killed, strains were considered low-virulent. The rationale for this variation was to reduce the number of animals used, in compliance with the

University of Florida Institutional Animal Care and Use Committee, while not compromising the integrity of the study.

Five two-week old ICR mice (Harlan Sprague, Indianapolis, Indiana) were used per bacterial isolate. Mice were challenged by over-load with iron-dextran (250 µg iron dextran/gram body weight of mouse) 2 h prior to bacterial inoculation. Broth cultures of *V. vulnificus* were grown for 4 h at 37°C in BHI to late log phase. Cultures were pelleted by centrifugation at 4400 x g for 15 min, resuspended in PBS, and adjusted by optical density of 0.64 at 420 nm. Mice received 0.5 mL intra-peritoneal injections of bacteria suspensions with approximately 1,000 bacteria. Mice were observed for a total of 48 h for mortality.

Virulence Assay Using Multiple Strains

The iron-overload mouse model was modified to test the fate of high-virulent and low-virulent strains when injected singly, or in mixed suspension. Bacterial strains were grown in 15 mL Brain Heart Infusion broth, centrifuged at 4000 x g for 15 min, washed in 10 mL PBS and repelleted by centrifugation. Cells were suspended in PBS to $\sim 10^8$ cfu/mL. Following serial dilution, inocula were prepared by combining 250 µL of four bacterial suspensions to produce a final concentration of 10^3 cfu/mL having an equal proportion of each strain. Groups of five ICR mice were injected intra-peritoneally with bacteria after iron over-load treatment. *Vibrio vulnificus* was enumerated in tail-blood at 30 min, 4, and 8 h time points. Blood was diluted in PBS, spread on TSA-1% plates, bacterial colonies enumerated, and confirmed by ELISA (150). Isolates were analyzed by PFGE for strain identification.

RESULTS AND DISCUSSION

The emphasis of this study was to identify phenotypic and/or genotypic profiling regimes that could effectively distinguish virulent strains of *V. vulnificus*, as well as their geographical source. The over-all effectiveness of the assays was judged by their reproducibility and whether clusters were produced that grouped strains by the desired characteristics.

Since *V. vulnificus* was described as a human pathogen, efforts to determine intraspecies variations in virulence have focused on phenotypic traits. Phenotypic characteristics have included: substrate metabolism (103, 104), colony morphotype (3, 135, 171), outer membrane protein and lipopolysaccharide constituents (5, 12), fatty acid composition (9), exoenzyme production (52, 53, 68, 83, 134, 167), virulence in animal models (73, 82, 135, 167, 171), and resistance to host immune responses (31, 56, 82, 152, 153, 171). Although early studies provided valuable information about the incidence and frequency of expressed characteristics among *V. vulnificus* strains, prediction of human pathogenicity using specific phenotypic properties was not beneficial. The focus of recent studies has shifted to incorporate genotyping techniques to determine diversity of *V. vulnificus* populations and predicting strain virulence. These techniques include analysis of RFLP profiles generated by PFGE, RT, PCR, and RAPD (8, 27, 29, 62, 162, 163).

Phenotypic and genotypic analyses used in these proceedings include FAME, biotype, capsule-type, LPS-type, PFGE, and RT. These techniques were chosen because of their proven utility, to determine the most effective means for clustering *V. vulnificus* isolates. The proportion of clinical and environmental isolates among defined clusters was used as a preliminary indicator.

FAME Analysis

Analysis of FAME profiles using the MIDI microbial identification system has proven utility for species identification of *V. vulnificus* (86); however, it has not been used to differentiate strain virulence. Results support the efficiency of this system for identifying *V. vulnificus* at the species level; however, discrimination at the strain level was inconsistent.

Fifty-six clinical isolates were tested to determine if known virulent strains demonstrated a high degree of similarity by FAME profile. All strains were identified as *V. vulnificus* (Euclidean value <10) and could be grouped into twelve FAME clusters by subspecies with a Euclidean value <6 (Figure 1) (127). Twenty-eight isolates (50%) grouped into cluster 9 (Table 3) and no strains showed identical profiles.

Three pair of isolates having similar clonal origin were tested: MO624-O and MO624-T, CVD713-O and CVD713-T, 1010-O and 1010-T. These isolates represent parental opaque strains (O) and spontaneous translucent daughter strains (T) (166). Although MO624-O and MO624-T were grouped within FAME cluster 9, the other related strains did not. CVD713-O and CVD713-T were in cluster 4 and 5, respectively, while 1010-O and 1010-T grouped in 2 and 9, respectively. These data show that it cannot be

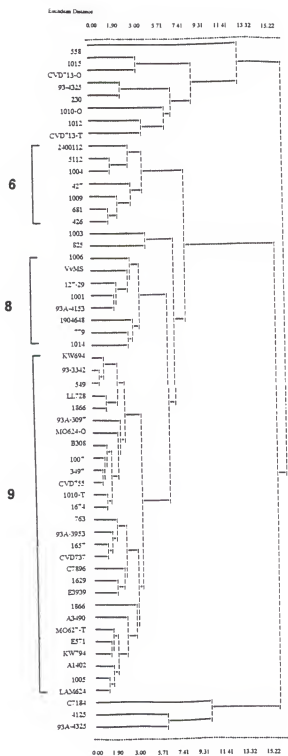


Figure 1: FAME dendrogram of clinical *Vibrio vulnificus* isolates

TABLE 3: Distribution of *Vibrio vulnificus* isolates among FAME clusters

FAME Cluster						
1	2	3	4	5	6	7
558	93-4325 230	1010-T	1015 CVD713-O	1012 CVD713-T	426 427 681 1004 1009 5112 2400112	825 1003

FAME Cluster						
8	9		10		11	12
1006	549	A1402	1010-O	C7184	93-4125	93-4325
779	763	A3490	93-3097			
1001	1005	B308	93-3497			
1014	1007	C7896	93-3953			
1904648	1629	CVD737	93-9942			
127-29	1657	CVD755	LAM624			
93-4153	1674	E3939	LL728			
VvMs	1806	KW694	MO624-O			
	1866	KW794	MO624-T			

assumed that opaque and translucent strains differ only by quantity of capsule, but differ by other physiological characteristics as well.

As a control, two colonies of strain 93A-4325 from the same blood agar plate were assayed. These isolates showed different FAME profiles, i.e.--clusters 3 and 12. Duplicate FAME experiments demonstrated excellent speciation capability; however, clustering below the subspecies level differed between trials.

Variability of profiles between isogenic opaque and translucent strains, between identical isolates run twice within the same experiment, and between identical strains in separate experiments, make FAME profiling less practical as a consistent means for subgrouping. Similar results were seen in experiments aiming to subgroup verotoxigenic strains of *E. coli* and *Salmonella enteritidis* (143). Variability of FAME profiles observed in repeat preparations from the same strain under standard conditions was as great as that observed between subgroups, limiting the usefulness of this technique as a discriminatory tool. Data from these experiments also substantiate that FAME profiles are easily affected by environmental factors, such as culture conditions; isolates with 100% similarity by ribotype and PFGE tested different by FAME profile (Table 4). Due to demonstrated variability of this technique in these and other studies, FAME profiles could not be considered a reproducible means for clustering *V. vulnificus*. Since only clinical isolates were used in FAME analyses, distinguishing a relationship between geography and FAME profile, and virulence and FAME profile, was not determined. Other studies have shown little if any difference in FAME profile of virulent and avirulent *V. vulnificus* isolates (9).

TABLE 4: *Vibrio vulnificus* strains with 100% similarity by RT and phenotype characteristics 43

Strains with 100% Ribotype Similarity	Ribotype	FAME	Biotype	Capsule type	LPS type	LD ₅₀
1005	A	9	B	1	3	-
1006	A	7	B	*	3	-
C7184	A	10	B	1	3	-
1002	B	-	C	7	1/5	25
1007	B	9	-	4	5	33
1009	B	6	-	4	1/5	2
E4125	B	-	B	4	1/5	6
CVD713-O	B	4	B	*	*	-
CVD713-T	B	5	C	*	*	-
CVD755	B	9	-	*	*	-
MO624-O	B	9	B	*	*	-
MO624-T	B	9	B	*	*	-
SEA10217	B	-	A	*	*	2
LL728	B	9	A	10	*	1
2796	B	-	-	*	*	-
60-80	B	-	-	*	*	-
825	B	2	B	*	*	6
1657	B	9	B	5	1	-
MLT271	B	-	-	*	*	-
MLT272	B	-	-	*	*	-
MLT285	B	-	-	*	*	-
MLT286	B	-	-	*	*	-
MLT287	B	-	-	*	*	-

- = no data

* = nontypeable

TABLE 4--continued

Strains with 100% Ribotype Similarity	Ribotype	FAME	Biotype	Capsule type	LPS type	LD ₅₀
MLT320	B	-	-	*	*	-
MLT322	B	-	-	*	*	-
MLT370	B	-	-	*	2	-
MLT375	B	-	-	*	*	-
KW794	C	9	C	*	*	8
KWE11	C	-	B	*	*	6
KWE12	C	-	B	*	*	-
KWG11	C	-	B	*	*	-
KWG12	C	-	B	*	*	-
MLT124	C	-	B	*	4	2100
MLT194	C	-	C	*	*	1400
MLT166	C	-	B	*	2	-
MLT201	C	-	-	*	4	nd
MLT200	C	-	B	*	3	-
MLT404	C	-	B	*	1	nd
MLT431	C	-	-	*	*	-
MLT436	C	-	-	*	*	-
MLT151	D	-	-	*	3	-
MLT190	D	-	-	*	3	-

- = no data

* = nontypeable

Biotype Analysis

Biotyping has proven capability in discriminating *V. vulnificus* biotype 1 from biotype 2 isolates. Biotype 1 is the principal group associated with human disease, and is different from biotype 2 by its ubiquity in the environment, inability to produce disease in eels, and positive indole production (11, 159). Biosca et al. (1996) have also demonstrated that biotype 2 strains are negative for mannitol metabolism, while biotype 1 strains are positive. In our studies, isolates were not tested for indole production, but all tested positive for mannitol utilization and are assumed to be biotype 1. Twenty-seven other substrates (e.g., cellobiose, D-fructose, L-alanine, and L-glutamic acid), tested positive for each *V. vulnificus* strain.

Ninety of 92 *V. vulnificus* isolates tested for biotype grouped within four clusters (A, B, C, and D) at 50% similarity index, which was arbitrarily chosen as the point to define clusters (Figure 2). Most isolates (77%) grouped within cluster B, with similar distribution between those of clinical (55%) and environmental (45%) origin (Tables 5 and 6).

Similar to results of FAME analysis, isogenic strains CVD713-O and CVD713-T with 100% PFGE and RT similarity, grouped separately into clusters B and D respectively (Table 4). Biotype variation of these strains resulted from differences in metabolism of 20 different substrates, yielding similarity index of approximately 40%. Four environmental isolates (KWE11, KWE12, KWG11, KWG12) with identical PFGE and RT profiles exhibited extreme heterogeneity in biotype even though they were located in biotype B (Figure 2). A single clinical isolate (KW794) identical by RT to the

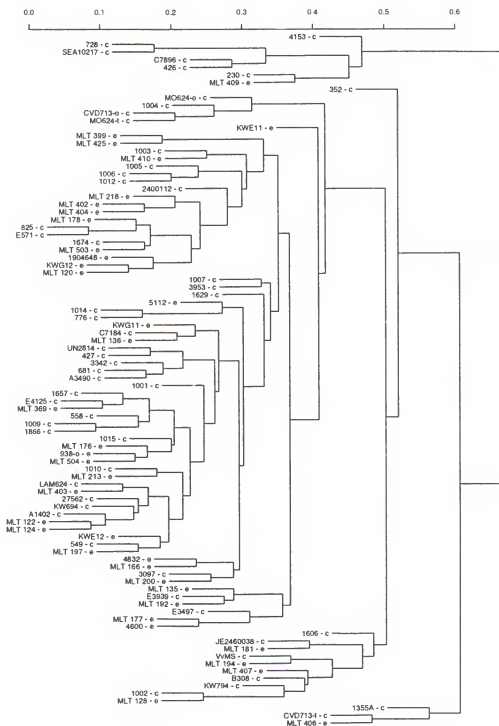


Figure 2: Biotype dendrogram of clinical and environmental *Vibrio vulnificus* isolates analyzed by binary metric, average linkage

TABLE 5: Distribution of *Vibrio vulnificus* isolates among biotype clusters

Biotype Cluster					
A	B			C	D
230	5112	MLT403	1629	1606	CVD713-T
426	1940648	MLT404	1657	1002	MLT406
4153	938-O	MLT410	1674	B308	
C7896	KWE12	MLT425	1866	JE2460038	
LL728	KWG11	MLT503	3097	KW794	
SEA10217	KWG12	MLT504	3342	VvMs	
MLT409	MLT120	427	3953	MLT128	
	MLT122	549	4600	MLT181	
	MLT124	558	4832	MLT194	
	MLT136	681	27562	MLT407	
	MLT138	776	2400112		
	MLT166	825	A1402		
	MLT176	1001	A3490		
	MLT177	1003	C7184		
	MLT178	1004	CVD713-O		
	MLT192	1005	E3497		
	MLT197	1006	E3939		
	MLT200	1007	E4125		
	MLT213	1009	E571		
	MLT218	1010	KW694		
	MLT369	1012	LAM624		
	MLT399	1014	MO624-O		
	MLT402	1015	MO624-T		
			UN2814		

TABLE 6: Distribution of clinical and environmental
Vibrio vulnificus isolates among biotypes

Biotype profile	A	B	C	D
Total # of isolates	7	71	10	2
% clinical isolates	86%	55%	60%	50%
% environmental isolates	14%	45%	40%	50%

aforementioned environmental isolates, was less similar (~50%) and grouped in biotype C (Table 4). No strains tested were 100% identical by biotype profile. In fact, the highest percent similarity was approximately 92% between strains MLT122 and MLT124. Also, environmental isolates from different geographical areas all grouped within cluster B.

Biotype variability among genetically identical isolates was prevalent in these studies, and reproducibility between replicate testing was low, with differences by up to 15 substrates per replicate. For these reasons biotype using the BiologTM microplate system could not be considered a reliable, reproducible, or practical technique for clustering *V. vulnificus* by phenotypic traits.

Serology Analysis

Serological reactivity is a proven means for identifying human pathogens from various environments. Recent studies examining clinical and environmental isolates of *V. vulnificus* show that strains pathogenic to eels (biotype 2) are distinguishable by LPS-type from those associated with human disease (biotype 1) (11). Antisera to indole-negative *V. vulnificus* strains are so selective that Biosca has proposed all isolates previously classified as biotype 2 to be identified as serovar E.

In the present study, capsular and LPS agglutination tests were performed on 234 isolates; 161 originating from environmental sources and 73 of clinical origin. Data are summarized in Tables 7, 8, and 9. Only 20% of all isolates tested agglutinated with any of the ten capsular antisera. Considerably fewer environmental isolates (8.6%) than clinical isolates (43.8%) were typeable, indicating greater diversity in their capsule

TABLE 7: Distribution of capsule serotype among clinical and environmental *Vibrio vulnificus* isolates

	Clinical Isolates	Environmental Isolates
Total # tested	73	161
Capsule-type		
1	6.90%	0.60%
2	8.20%	-
3	8.20%	1.90%
4	5.50%	-
5	2.70%	1.20%
6	1.40%	-
7	4.10%	2.50%
8	1.40%	0.60%
9	2.70%	1.20%
10	2.70%	0.60%
non-typeable	56.20%	91.40%

TABLE 8: Distribution of LPS serotype among
clinical and environmental *Vibrio vulnificus* isolates

	Clinical isolates	Environmental isolates
Total # tested	73	151
LPS-type		
1	5.5%	0.7%
2	20.5%	15.3%
3	8.2%	13.4%
4	11.0%	14.9%
5	2.7%	-
1/5	11.0%	2.0%
1/4	-	0.7%
2/3	-	0.7%
nontypeable	41.1%	52.3%

TABLE 9: Proportions of *Vibrio vulnificus* isolates typed by capsule or LPS,
by capsule and LPS, or nontypeable

	Clinical isolates	Environmental isolates
Total # tested	73	161
% typed by capsule or LPS	45%	43.2%
% typed by capsule and LPS	29%	4.6%
% not typed by either capsule or LPS	26%	52.2%

composition. The most common capsule types among clinical isolates were types 2 and 3, with six strains (8.2%) each.

Five anti-LPS-antisera were used to test *V. vulnificus* strains. LPS serology proved more successful than capsule, with 51.3% of all isolates being agglutinated. More environmental isolates (47.7%) were typed by LPS compared to capsule-type; 58.9% of clinical isolates reacted with LPS antisera. Among clinical isolates typed, 20.5% were identified as capsule-type 2; 17.2% of environmental strains were identified as type 2.

Of environmental isolates tested, 43.2% could be typed by either capsule or LPS, 52.2% were not typed in either serological assay, and only 4.6% were identifiable by both (Table 9). Forty-five percent of clinical isolates were identified by either capsule or LPS, 26% could not be typed using either technique, and 29% were typed by both capsule and LPS (Table 9).

High percentages of both clinical and environmental *V. vulnificus* strains were not typed by capsular and/or LPS antisera; this suggests that *V. vulnificus* populations exhibit a high diversity of cell surface epitopes. Other studies have shown similar percentages of non-typeable isolates, but have shown no correlation between serotype and virulence of *V. vulnificus* (39, 130). A notable result from these studies is that more clinical isolates (29%) were identifiable by both capsule-type and LPS-type than environmental strains (4.6%) (Table 9). Serological tests using combinations of capsular and LPS antisera may prove an effective means for identifying virulent *V. vulnificus* strains. Also in these experiments, isolates having identical RT profiles tested different by capsule and LPS type (Table 4). Based on the high percentage of non-typeable strains in these experiments,

serological identification was not a reliable means to differentiate *V. vulnificus* isolates by virulence, geography, or environmental niche.

PFGE Analysis

PFGE has become an important tool in epidemiological research of bacterial disease outbreaks, as well as for discrimination among isolates. In epidemic and sporadic outbreaks, PFGE has been used to identify etiological strains including *Escherichia coli* O157:H7, *Vibrio cholerae* O1, *Aeromonas hydrophila*, *Yersinia enterocolitica*, *Legionella pneumophila*, and *Propionibacteria* (16, 27, 50, 91, 128, 146, 147). For example, PFGE has been shown to effectively differentiate antibiotic resistant mutants from parent strains (114), and is capable of differentiating *V. tapetis* from other *Vibrio* spp. (32). Food borne outbreaks of listeriosis in France have utilized PFGE to trace illnesses to the source of infection (66). PFGE using *ApaI* restriction endonuclease has proven useful for differentiating *Listeria monocytogenes* serovar 4b associated with human listeriosis outbreaks, from other serotypes of different environmental origin (15, 24).

In the present study, 34 clinical and environmental *V. vulnificus* isolates were analyzed by PFGE using a variety of endonucleases. *SfiI* and *NotI* were subsequently used for similarity analysis because resulting profiles generated an appropriate number of restriction fragments (14 - 28) with optimum distribution. Binary metric average linkage analysis showed that most strains tested were less than 40% similar using *NotI* or *SfiI* endonuclease (Figures 3 and 4). Four clinical strains associated with Louisiana cases in the early 80's exhibited higher similarity relative to other strains. Specifically, 1005 and 1006 were >80% similar when compared using *NotI*, while 1007 and 1009 were

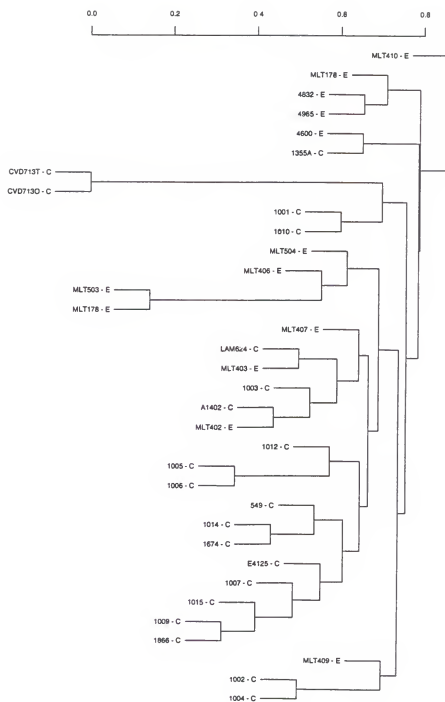


Figure 3: Dendrogram of clinical and environmental *Vibrio vulnificus* isolates restricted with *NotI* endonuclease and typed by PFGE

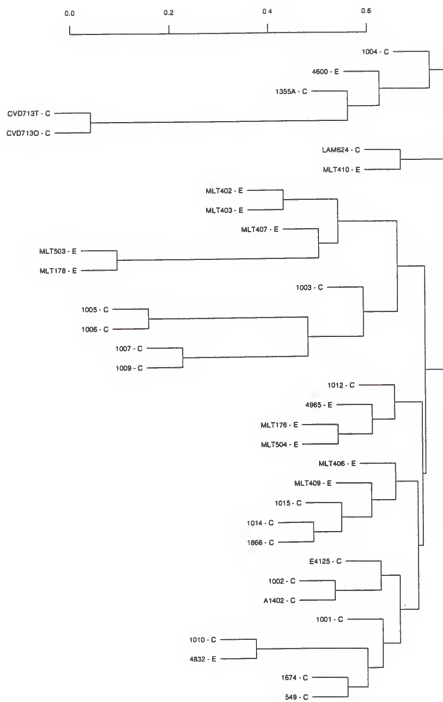


Figure 4: Dendrogram of clinical and environmental *Vibrio vulnificus* isolates restricted with *Sfi*I endonuclease and typed by PFGE

approximately 75% similar. Only strains CVD713-O and CVD713-T had identical profiles by *NotI* restriction, and differed only by one band by *SfiI* profile. Complete similarity, as demonstrated by *NotI* digestion, was not unexpected for these isogenic strains; however, the difference demonstrated by *SfiI* restriction is also not surprising knowing that CVD713-T was genetically changed by transposon mutagenesis.

Studies performed by Buchreiser et al. (1995) have described the diversity of *V. vulnificus* populations in single oysters. Likewise, studies by Singer et al. (1994) have used PFGE to show that *V. vulnificus* populations in the environment do not share clonal origin (136). These previous studies support the findings of the present experiments. Even though reproducibility was excellent, *V. vulnificus* isolates demonstrated tremendous diversity. This diversity was expected, but could be more useful if isolates sharing similar phenotypic characteristics, such as serotype, were assayed collectively. Low similarity among strains made identifying clusters ineffective, and PFGE did not correlate with virulence. However because of its high discriminatory power, PFGE was ideal for retrospective epidemiological investigations, and was used in further studies.

Ribotype Analysis

Ribotyping is known to distinguish bacterial strains, but is usually less discriminatory than PFGE (155). Two RTs have been identified for *V. tapetis*, which is responsible for brown ring disease of cultured clams (32). In early reports by Aznar et al. (1993), RT profiles were described for a relatively small number of *V. vulnificus* strains. In their experiments, strains belonging to biotype 2 were more homogeneous and therefore distinguished by RT than biotype 1 strains.

In these studies, 226 *V. vulnificus* isolates were profiled by ribotype using five restriction enzymes. Of enzymes tested, *Hind*III was used for binary metric, average linkage because the profiles with fewer, yet resolvable bands (11 to 18) (Figure 5). At a similarity index of 50%, nine clusters were identified that contained the majority of isolates. Clusters were assessed for distribution of isolates with different geographical origin, environmental source, and phenotype (i.e. FAME, biotype, serotype, and virulence).

Of 226 *V. vulnificus* isolates ribotyped, 222 clustered in nine RTs (A-I) (Figure 6). Eighty-seven percent of all isolates grouped within three clusters (B, C, and D). Distribution of clinical and environmental isolates among the cluster is shown in Tables 10 and 11. Cluster B had a similar proportion of clinical (51%) and environmental isolates (49%). In contrast, clusters C and D contained more environmental (85% and 68%, respectively) than clinical isolates (15% and 32%, respectively).

Source of isolation was designated by body of water (Atlantic, Pacific, or Gulf of Mexico waters) or environmental sample (seawater, shellfish, or sediment). Geographical distribution of environmental isolates is shown in Table 12. Fifty-six percent of isolates in RT B were from Pacific waters, 27% were from the Gulf of Mexico, and 17% from Atlantic waters. Isolates in RT C and B had similar geographical distribution, 55% and 51% isolates were from the Gulf of Mexico, 43% and 43% were from Atlantic waters, and 2% and 6% were from the Pacific, respectively. *Vibrio vulnificus* isolates, collected from shellfish, sediment, and seawater, were equally dispersed between clusters B, C, and D (Table 13). Two isolates collected from a saltwater aquaculture pond in Hawaii grouped in cluster B; in fact, the majority of environmental isolates in cluster B were from

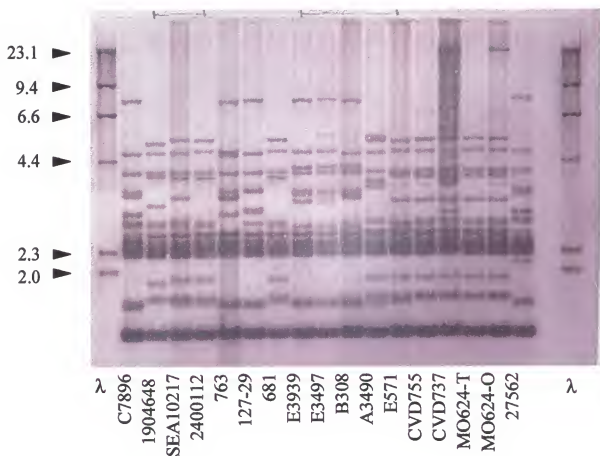


FIGURE 5: Ribotype of *Vibrio vulnificus* isolates restricted with *Hind*III

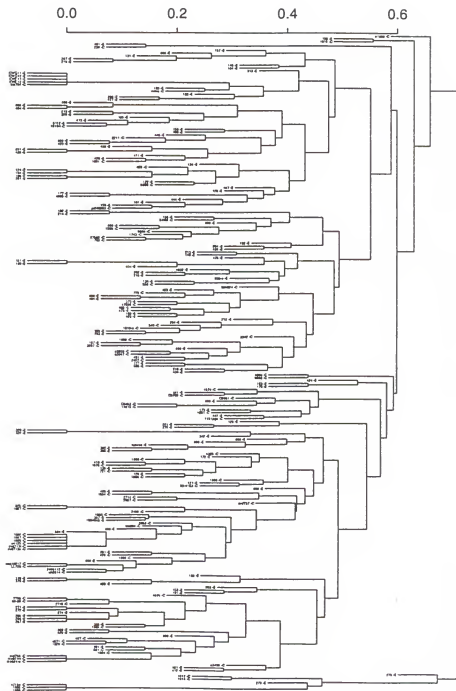


Figure 6: Dendrogram of clinical and environmental *Vibrio vulnificus* isolates by RT

TABLE 10: Distribution of *Vibrio vulnificus* isolates among RT clusters

Ribotype Cluster					
A	B			C	
MLT273	MLT120	MLT375	1769	MLT122	MLT406
1005	MLT121	MLT385	1866	MLT124	MLT408
1006	MLT135	MLT386	1976	MLT126	MLT411
1012	MLT153	MLT391	1984	MLT131	MLT413
1014	MLT155	MLT392	1985	MLT133	MLT423
C7184	MLT172	MLT402	2490	MLT134	MLT426
	MLT181	MLT410	2714	MLT148	MLT427
	MLT191	MLT412	2715	MLT150	MLT430
	MLT195	MLT425	2798	MLT159	MLT431
	MLT203	MLT429	2957	MLT161	MLT436
	MLT268	MLT504	3953	MLT163	MLT444
	MLT269	1904648	4325	MLT166	MLT445
	MLT271	LASWOC	2400112	MLT177	MLT447
	MLT272	407	60-80	MLT184	MLT448
	MLT274	426	93-4153	MLT189	4965
	MLT281	427	A3490	MLT194	5112
	MLT285	681	CVD713-O	MLT196	KWE11
	MLT286	721	CVD713-T	MLT197	KWE12
	MLT287	825	CVD737	MLT200	KWG11
	MLT320	1002	CVD755	MLT201	KWG12
	MLT322	1003	E571	MLT207	352
	MLT342	1004	E4125	MLT209	1001
	MLT362	1007	KW694	MLT210	1010-T
	MLT365	1009	LL728	MLT219	2211
	MLT364	1355A	MO624-O	MLT220	B308
	MLT367	1459	MO624-T	MLT313	JE2460038
	MLT369	1629	SEA10217	MLT404	KW794
	MLT370	1657	UN2814		VvMs

TABLE 10--continued

Ribotype Cluster						
D		E	F	G	H	I
MLT127	MLT464	MLT173	MLT401	406	MLT123	MLT128
MLT136	MLT465	MLT403	230	9006	MLT176	MLT221
MLT149	MLT474	MLT441				MLT270
MLT151	MLT475	MLT442				
MLT158	MLT478	1591				
MLT169	MLT491	1674				
MLT182	MLT493	14015				
MLT186	MLT508	1121PGC				
MLT187	4832	C8453				
MLT190	938-O	C8769				
MLT192	549	C8961				
MLT202	549M					
MLT204	558					
MLT212	763					
MLT213	779					
MLT214	1010-O					
MLT215	1596					
MLT218	1606					
MLT284	1743					
MLT373	2457					
MLT399	3097					
MLT428	3342					
MLT434	9000					
MLT439	27562					
MLT440	C7896					
MLT446	E3947					
MLT458	E3939					
MLT459	LAM624					

TABLE 11: Distribution of clinical and environmental
Vibrio vulnificus isolates among RT clusters

Ribotype profile	A	B	C	D	E	F	G	H	I
Total # of isolates	6	85	55	56	11	2	2	2	3
% clinical isolates	17%	51%	15%	68%	36%	50%	100%	-	100%
% environmental isolates	83%	49%	85%	32%	64%	50%	-	100%	-

TABLE 12: Geographical distribution of environmental
Vibrio vulnificus among RT clusters

Ribotype profile	A	B	C	D	E	F	G	H	I
No. isolates per cluster	1	41	47	38	4	1	0	2	3
% from Atlantic waters	-	17%	43%	43%	-	-	-	50%	67%
% from Pacific waters	100%	56%	2%	6%	-	-	-	-	33%
% from Gulf of Mexico waters	-	27%	55%	51%	100%	100%	-	50%	-

TABLE 13: Distribution of *Vibrio vulnificus* isolates
from environmental niches among RT clusters

Ribotype profile	A	B	C	D	E	F	G	H	I
No. of environmental isolates	1	41	47	38	4	1	0	2	3
% shellfish isolates	100%	42%	51%	38%	25%	-	-	50%	33%
% sediment isolates	-	22%	32%	41%	50%	100%	-	50%	-
% seawater isolates	-	34%	17%	19%	25%	-	-	-	67%
% pond water isolates	-	2%	-	-	-	-	-	-	-

Hawaiian sources. For cluster B, 42% were of shellfish origin, 22% sediment, 34% seawater and 2% pondwater. Fifty-one percent in cluster C were from shellfish, 32% from sediment, and 17% seawater. Cluster D isolates were 38% shellfish, 41% sediment, and 19% seawater. Distribution by source and ribotype is shown in Table 13. These data indicate that RT may be related to geographical source, but not environmental niche.

Distribution of phenotypes by RT is given in Table 4. Isolates from FAME cluster 9 and biotype cluster B were found in RT clusters B, C, and D. Likewise, no specific capsule- or LPS-type was associated exclusively with RT B, C, or D.

Six groups of clinical isolates showed 100% similarity by RT profile (Table 14); three groups contained two isolates, two groups contained three isolates, and one group contained six isolates. Nine groups of environmental isolates had 100% RT similarity (Table 14); one group contained three isolates, and the other eight contained two. A single group of five identical isolates contained one clinical and four environmental strains. All strains having identical RT demonstrated different FAME and biotype profiles; however, clinical isolates with 100% RT similarity belonged to LPS-serotypes 1, 5, 3, or 1/5 (Table 14).

This study provides further evidence of the genetic diversity of *V. vulnificus* populations, and concurs with other investigators studying *HindIII* generated RT profiles (62). Due to observed differences in distribution of clinical and environmental isolates among RT B and C, strains were selected to determine if strain virulence correlated with RT.

TABLE 14: Distribution of *Vibrio vulnificus* isolates among phenotype profiles and RTs

FAME Profile	Ribotype								
	A (6)	B (85)	C (55)	D (56)	E (11)	F (2)	G (2)	H (2)	I (3)
1	-	-	-	1	-	-	-	-	-
2	-	1	-	-	1	-	-	-	-
3	-	-	-	1	-	-	-	-	-
4	-	1	-	-	-	-	-	-	-
5	1	1	-	-	-	-	-	-	-
6	-	6	1	-	-	-	-	-	-
7	-	2	-	-	-	-	-	-	-
8	2	2	2	1	-	-	-	-	-
9	1	13	2	9	1	-	-	-	-
10	1	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-
12	-	1	-	-	-	-	-	-	-

Biotype									
A	-	4	-	1	-	1	-	-	-
B	5	27	13	16	3	-	-	1	-
C	-	2	5	1	-	-	-	-	-
D	-	1	1	-	-	-	-	-	-

Capsule-type									
1	2	1	-	-	1	-	-	-	-
2	-	1	-	2	-	-	-	-	-
3	1	3	1	1	-	-	-	-	-
4	-	4	-	-	-	-	-	-	-
5	-	1	-	-	-	-	-	-	-
6	-	-	1	-	-	-	-	-	-
7	-	1	3	-	-	-	-	-	-
8	-	-	1	1	-	-	-	-	-
9	-	2	-	2	-	-	-	-	-
10	-	1	-	1	-	-	-	-	-

TABLE 14--continued

LPS-type	Ribotype								
	A (6)	B (85)	C (55)	D (56)	E (11)	F (2)	G (2)	H (2)	I (3)
1	-	2	2	-	-	-	-	-	-
2	1	2	11	10	2	-	-	1	-
3	3	1	6	5	-	1	-	1	-
4	-	5	10	4	-	-	-	-	-
5	-	1	-	-	-	-	-	-	-
1/5	-	7	-	-	-	-	-	-	-
1/4	-	-	1	-	-	-	-	-	-
2/3	-	-	-	-	-	1	-	-	-
2/4	-	1	-	-	-	-	-	-	-
non-typeable	2	66	25	37	9	0	2	-	3

Ribotype and LD₅₀ Analyses

A total of 37 isolates were assayed for LD₅₀, 18 from RT cluster B and 19 from RT cluster C (Tables 15 and 16). A pronounced difference in virulence existed between the two clusters. The mean LD₅₀ for isolates in clusters B and C were approximately 40 and 2000 cfu, respectively. The difference in mean LD₅₀ for clinical isolates from cluster B (8 cfu) was almost 600 times lower than that of cluster C (4600 cfu). Of 22 environmental isolates tested, 13 (59%) were not virulent at the highest challenge dose (10³ cfu). All clinical strains tested were virulent in mice at $\leq 10^3$ cfu.

Even though a marked difference in mean LD₅₀ was observed between RT B and C, high-virulence strains did not group exclusively into either cluster. In fact, isolates KW794 and KWE11 from a Key West case described later, grouped in cluster C and exhibited extremely low LD₅₀ values (5 and 8 respectively). For this reason RT should not be considered an absolute means for discriminating high- and low-virulence strains.

If phenotypic and genotypic characteristics cannot be used to accurately determine virulence of *V. vulnificus*, future research should focus on developing virulence assays which select for high-virulence strains. Results in case-studies and mixed-isolate studies (described below), show that the iron-overload mouse model may serve as an accurate tool for understanding and predicting *V. vulnificus* virulence.

Case Studies

The discriminatory power of PFGE was used in case studies to identify the vehicle of *V. vulnificus* infection, characterize the number and types of *V. vulnificus* strains found

TABLE 15: LD₅₀ values of *Vibrio vulnificus* isolates for RT cluster B

Ribotype B Isolates	LD ₅₀
Vv426	4
Vv825	6
Vv1002	25
Vv1003	2
Vv1007	33
Vv1009	2
E4125	6
LL728	1
2400112	3
SEA10217	2
MLT281	5
MLT362	175
MLT364	145
MLT365	>10 ⁴
MLT367	>10 ⁴
MLT403	>10 ⁴
MLT504	130

TABLE 16: LD₅₀ values of *Vibrio vulnificus* isolates for RT cluster C

Ribotype C Isolates	LD50
KW794	8
JE2460038	1500
Vv352	12,300
KWE11	5
MLT122	30
MLT124	2100
MLT131	>10 ⁴
MLT134	>10 ⁴
MLT194	1400
MLT197	>10 ⁴
MLT200	>10 ⁴
MLT201	>10 ⁴
MLT207	220
MLT209	>10 ⁴
MLT401	200
MLT404	>10 ⁴
MLT406	>10 ⁴
MLT436	>10 ⁴
MLT448	>10 ⁴

in patient tissues, and case-associated oysters. Such information could provide better approaches to risk management.

The following narrative details four cases of *V. vulnificus* disease. Estimates of the infective dose are made for two cases, and PFGE analysis of strains from human and oyster specimens are discussed. Although blood and oysters specimens were not collected from all cases, each case is relevant to this study by demonstrating either the predominance of a single *V. vulnificus* strain in post-mortem patients, or the difficulty of isolating strains from shellfish which are genetically identical to those producing human infection.

Case #1 occurred in Bay County, Florida. During June of 1994, a 26-year old female with preexisting diabetes became ill less than 24 hours after eating an unknown number of raw oysters. Primary sepsis resulted in death in less than 24 hours after admittance to the hospital. All blood isolates were identified as *V. vulnificus* and were present at 8.8×10^7 cfu/mL blood. PFGE analyses using four different restriction enzymes showed that all 50 blood isolates had identical fragment profiles (Figure 7). Oysters associated with this case could not be obtained from the implicated restaurant or state regulatory agency.

Case #2 occurred in Key West, Florida. During July of 1994, a 25-year old male with preexisting hepatitis acquired a *V. vulnificus* infection less than 24 hours after eating six raw oysters. Death occurred from primary sepsis less than 24 hours after hospital admission. All blood isolates were identified as *V. vulnificus* and were present at 1.1×10^8 *V. vulnificus*/mL. PFGE profiles of 50 isolates showed 100% homology using four restriction enzymes (Figure 8).

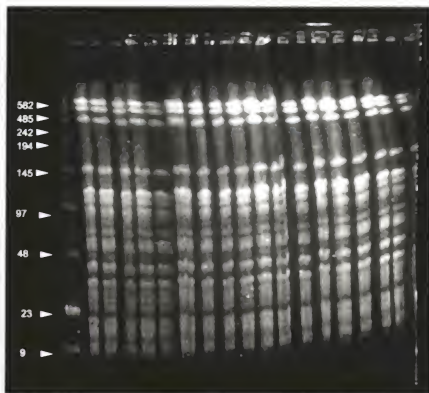


Figure 7: PFGE profiles of *Vibrio vulnificus* blood isolates from Case #1

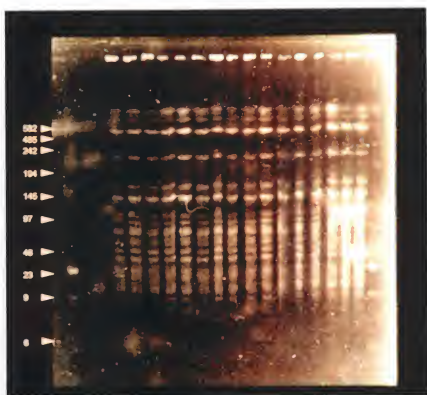


Figure 8: PFGE profiles of *Vibrio vulnificus* blood isolates from Case #2

Oysters obtained from the implicated restaurant, corresponding to a single implicated sample harvest lot number, were analyzed to determine both concentration and genetic diversity of *V. vulnificus* isolates. Oysters were harvested five days before onset of disease, and were refrigerated at the restaurant prior to analysis. The total *V. vulnificus* level was 9.6×10^3 /g oyster, indicating the patient ingested approximately 6×10^5 total *V. vulnificus*.

Using the direct-plating procedure, 29 *V. vulnificus* colonies were collected from oysters, and subsequent PFGE analysis revealed eight unique profiles. DNA of nine strains were totally or partially degraded. Four of the 29 isolates had PFGE profiles identical to that of the clinical strain, with each of four restriction enzymes used (Figure 9). LD₅₀ tests were performed for strains representing each of the eight different PFGE profiles, and the blood isolate. Importantly, only the clinical strain (LD₅₀ = 7 cfu) and case-matched oyster strain (LD₅₀ = 5 cfu) were virulent in the mouse model. All other oyster isolates were not lethal at 10^4 cfu per mouse.

The pathogenic strain was present in implicated oyster meat at 2×10^3 /g meat. Therefore, based on ingestion of six oysters we estimated that the patient consumed approximately 1×10^5 cfu of the virulent strain.

Case #3 occurred in Cocoa Beach, Florida during September of 1994. A 45-year old male with alcohol cirrhosis of the liver died from *V. vulnificus* septicemia less than 24 hours after eating four or five raw oysters. The hospital laboratory did not supply a blood sample but provided a single isolate. The implicated oysters were harvested one-day before ingestion. The total *V. vulnificus* level in oysters was 4.3×10^4 /g meat. PFGE

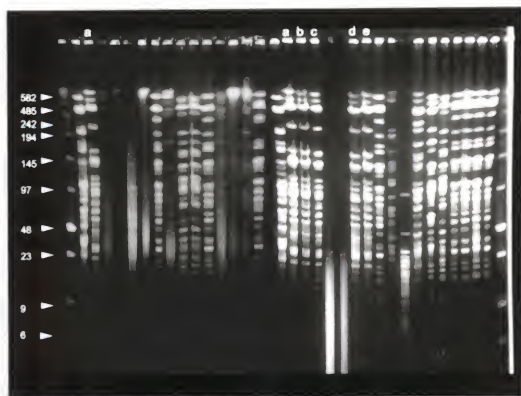


Figure 9: PFGE profiles of *Vibrio vulnificus* oyster isolates from Case #2

analysis of 67 oyster isolates produced 18 different profiles, none of which matched that of the clinical isolate (data not shown). In this case, we estimated that the patient ingested approximately 2×10^6 total *V. vulnificus*.

Case #4 occurred in Bay County, Florida. During November of 1994, a 36-year old male with alcoholic cirrhosis of the liver died of primary sepsis after contaminating a wound on his hand while cleaning fish. Patient blood contained 2.0×10^{10} *V. vulnificus*/mL. All blood isolates were *V. vulnificus* and showed identical PFGE profiles using four different restriction enzymes (Figure 10).

Previous reports of *V. vulnificus* infections have described the pathology of disease, but not the level of a specific virulent strain in shellfish or human tissue. The present study is the first to: 1) measure genotype polymorphism of multiple patient isolates, 2) identify the PFGE profile of the case strain in the food vehicle, and 3) measure the concentration of the specific case strain in the food vector.

All evidence from this study indicates that *V. vulnificus* infections result in proliferation of a single pathogenic strain. Analysis of over 150 total blood isolates showed a single PFGE profile in each of three human infections. RT tests show similarity indexes for these clinical specimens to be <50% (Figure 6). This finding supports the independent action or single-organism hypothesis described by Rubin (124), where within mixed populations "each bacterium acts alone or is independently capable of introducing infection." Further evidence in support of this hypothesis has been presented in studies using PFGE analysis of multiple *E. coli* isolates from septicemic patients (7, 89, 90). In these studies, all blood isolates had identical PFGE profiles with the exception of one

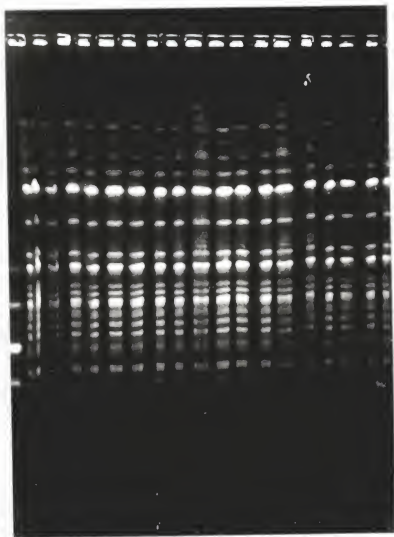


Figure 10: PFGE profiles of *Vibrio vulnificus* oyster isolates from Case #4

isolated from a cutaneous lesion. It would be important to similarly examine *V. vulnificus* isolates from both blood and wound tissues of individuals who develop septicemia via wound contamination, and determine if multiple strains are present.

These results also indicate that high-risk individuals are susceptible to relatively low concentrations of *V. vulnificus*. In one case, the concentration in implicated oysters was as low as 2×10^3 cfu/g, yet levels in Gulf of Mexico oysters can commonly reach 10^5 /g (151). Importantly, we observed that this concentration in environmental samples was associated with incidence of human disease.

This information strengthens the hypothesis that iron-overload mouse models accurately represent the disease process of *V. vulnificus* in humans. This is supported by case #2 where only the oyster isolate with a PFGE profile identical to the human isolate (among eight different PFGE types) was virulent in mice. These case studies show that human death from *V. vulnificus* septicemia results from single high-virulence strains proliferating in blood, supporting the independent-action hypothesis described by Rubin (124). This finding is significant because it may indicate a potential for implementing new regulatory regimens restricting shellfish harvest according to levels of high-virulence *V. vulnificus* strains rather than total counts. But for these regulations to be of value to industry, it must be determined that infections are initiated exclusively by high-virulence strains and not heterogeneous mixtures with varying virulence.

Virulence Assay Using Multiple Strains

Knowing that human mortality results from single *V. vulnificus* strains in human blood, studies were performed to ascertain whether infection can be initiated by multiple or single strains of *V. vulnificus*.

Mice were inoculated with one of two heterogeneous bacterial mixtures. Each contained 4 strains of *V. vulnificus* with different PFGE profiles and LD₅₀ values (Table 17). Mixture #1 contained three low-virulence strains and 1 high-virulence; Mixture #2 contained two low-virulence strains and two high-virulence. PFGE profiles of bacterial isolates collected from each set of mice are shown in Figures 11, 12, and 13.

Figure 11 shows isolates collected from mice inoculated with Mixture #1, 30 minutes, 4 hours, and 8 hours post-inoculation. Isolates from each time-point had identical PFGE profiles matching the high-virulence strain present in the inoculum (Table 17).

Figure 12 shows PFGE profiles of isolates collected from mice inoculated with Mixture #2, 30 minutes after bacterial challenge. These early time-point isolates confirm the presence of all four *V. vulnificus* strains in mouse blood (Table 17).

Figure 13 shows PFGE profiles of isolates collected 8 hours (post-mortem) after inoculation with Mixture #2. Only two PFGE profiles persisted in blood collected at this last time-point. All profiles matched those of the two high-virulence strains; of these isolates, 77% were the most virulent strain (Vv 1003) (Table 17). This is that virulent strains may demonstrate an increased capability to proliferate in blood, since Vv1003 represented only 30% of cells in the original inoculum.

TABLE 17: Multi-strain virulence tests of *Vibrio vulnificus*

			% presence in blood		
Strain Mix #1	LD ₅₀	% presence in inoculum	30 min	4 hr	8 hr
Vv-Rc2	5 cfu	12%	100%	100%	100%
Vv-Rc3	>10 ⁶ cfu	26%	-	-	-
Vv-Rc7	>10 ⁶ cfu	44%	-	-	-
Vv-Rc25	>10 ⁶ cfu	18%	-	-	-

			% presence in blood		
Strain Mix #2	LD ₅₀	% presence in inoculum	30 min	4 hr	8 hr
Vv1003	2 cfu	30%	43%	37%	77%
VvJe246003	1500 cfu	13%	14%	16%	23%
MLT403	>10 ⁶ cfu	19%	14%	21%	-
MLT404	>10 ⁶ cfu	39%	29%	26%	-



Figure 11: PFGE profiles of *Vibrio vulnificus* isolates from multi-strain virulence assays (Mixture #1) 8 hours post-inoculum



Figure 12: PFGE profiles of *Vibrio vulnificus* isolates from multi-strain virulence assays (Mixture #2), 30 minutes post-inoculum



Figure 13: PFGE profiles of *Vibrio vulnificus* isolates from multi-strain virulence assays (Mixture #2) 8 hours post-inoculum

From this study it was determined that *V. vulnificus* disease may begin with multiple strains from a population; but ultimately, high-virulence strains predominate in host tissue resulting in death. This finding supports the observations of a single high-virulent strain in human case studies, and also invalidates the measurement of high-virulence strains in shellfish to predict human risk. Evidence from this study indicates that low-virulence strains in shellfish may be involved with initiating disease. Most importantly, these experiments further strengthen the validity of the mouse model as a means for predicting *V. vulnificus* disease in humans.

Seasonal Studies

Vibrio vulnificus disease is known to occur in warm months of the year. This could result from an increase in bacterial number, or a proportional increase in specific high virulence strains. Therefore, the objective of this study was to determine if a relationship exists between season and levels of virulent *V. vulnificus* strains in oysters. *Vibrio vulnificus* isolates used in seasonal studies were collected from Apalachicola Bay during April, July, and October of 1995. Harvest conditions and *V. vulnificus* concentrations, determined by MPN, are given in Table 18. For oysters harvested in April, the month generally accepted as the beginning of "*V. vulnificus* season," levels were $1.8 \times 10^3/\text{g}$ oyster meat. Summertime oysters generally harbor higher levels of *V. vulnificus* than shellfish harvested during any other time of the year; however, levels were lower than usual during this particular time interval at $5.3 \times 10^2/\text{g}$ oyster meat. By October, concentrations had dropped to $3.6 \times 10^1/\text{g}$ meat.

TABLE 18: Environmental parameters of seasonal studies: water temperature, salinity, and *Vibrio vulnificus* concentrations

	Spring	Summer	Fall
water temperature (Celcius)	26.5	28.7	24.4
water salinity (parts per thousand)	19.7	30	30.9
<i>V.vulnificus</i> concentration in oyster sample (VV/g)	3×10^3	5.3×10^2	3.6×10^1

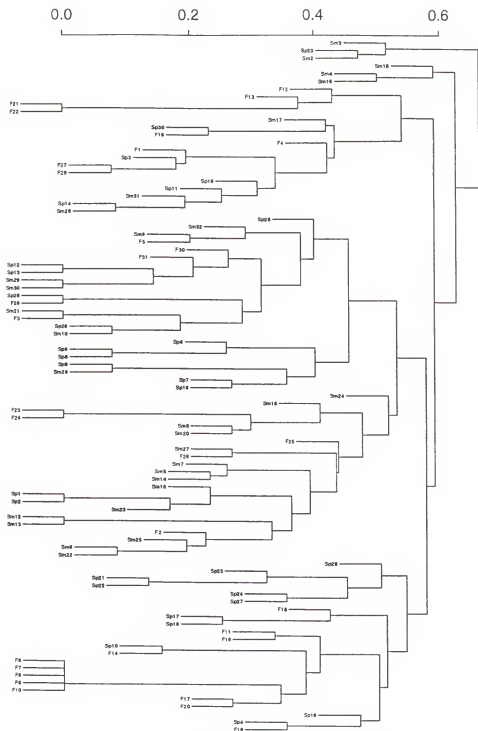


Figure 14: Dendrogram of seasonal *Vibrio vulnificus* isolates by RT

Thirty *V. vulnificus* isolates from each season were randomly selected by direct-plating technique, and assayed for RT with *Hind*III. Similarity of seasonal isolates among RT profiles is given in Figure 14. All but three isolates grouped in nine clusters at 50% similarity index; the distribution of isolates among clusters is given in Table 19. Seventy-nine percent of all isolates grouped in four RT (i.e., 4, 5, 6 and 9). Of the 23 isolates in cluster 4, 47.8% were from springtime oysters, whereas two-thirds of the 21 isolates in cluster 5 were from shellfish harvested during summer months. Eleven of 14 isolates in cluster 9 were collected from October oysters, while no strains in this grouping were summertime isolates. Only ribotypes 2, 3, and 7 had isolates exclusively from one season, but these were present in small numbers.

Isolates from each season were tested for virulence (Table 20). Strains were considered virulent if 50% of a population were killed by $\sim 10^3$ *V. vulnificus* cells; if not, they were considered low-virulent. A seasonal high for virulent isolates was observed with spring isolates (92%), and decreased with summer isolates (45%), and fall isolates (33%). Chi-square test of independence was applied to virulence data, yielding $\chi^2 = 9.057$ and $p = 0.011$. This indicates a significant relationship between virulence and season of isolation. It may be that virulent *V. vulnificus* strains multiply at faster rates when environmental conditions become more favorable in the early spring. This would explain the higher incidence of virulent isolates in spring oysters. However, knowing that mixtures of high- and low-virulent *V. vulnificus* strains may function synergistically to initiate infection, and that high-virulent strains were not shown to predominate in case-associated oysters, it is not safe to assume that low levels of virulent strains equals low

TABLE 19: Distribution of seasonal *Vibrio vulnificus* isolates among RT clusters

Ribotype Profile #	1	2	3	4	5	6	7	8	9
Total # of isolates	2	2	4	13	23	21	5	3	14
# of spring isolates	1	-	-	5	11	2	5	2	3
# of summer isolates	1	2	-	3	7	14	-	-	-
# of fall isolates	-	-	4	5	5	5	-	1	11

TABLE 20: Virulence of *Vibrio vulnificus* isolates from seasonal studies

Strain Designation	Virulence (+/-)	Strain Designation	Virulence (+/-)	Strain Designation	Virulence (+/-)
Sp1	+	Sm5	-	F1	+
Sp2	+	Sm9	+	F2	+
Sp10	+	Sm10	-	F3	+
Sp12	+	Sm12	-	F5	-
Sp13	+	Sm13	-	F6	-
Sp14	-	Sm16	-	F7	-
Sp15	+	Sm17	+	F8	-
Sp16	+	Sm21	+	F9	-
Sp18	+	Sm23	+	F10	-
Sp22	+	Sm26	+	F11	+
Sp25	+	Sm28	-	F17	+
Sp29	+			F18	-
				F23	-
				F24	-
				F27	-
92% virulent		45% virulent		33% virulent	

+ = virulent

- = nonvirulent

 $p = 0.011$

human risk. Total levels should continue to be assayed to determine the potential for human disease.

Effect of Elevated Storage Temperature on Levels of Virulent *V. vulnificus* Strains in Oysters

The intent of storage studies was to define the distribution of virulent *V. vulnificus* strains in oysters subjected to different post-harvest temperatures. Studies have shown that concentrations of *V. vulnificus* increase in oysters left at ambient air temperatures for 36 hours (37). *Vibrio vulnificus* isolates were collected from fresh oysters, and those subjected to temperature abuse at 20 and 27°C for 24 h. The mean concentrations of *V. vulnificus* in duplicate oyster samples are given in Table 21.

A decrease was noted in levels of *V. vulnificus* stored at elevated temperature; i.e.-- from 1.6×10^6 Vv/g meat in fresh shellfish, to 1.4×10^4 and 9.0×10^3 Vv/g meat in those stored at 20°C and 27°C respectively. This data contradicts findings reported by Cook (37). PFGE profiling was used to differentiate isolates for subsequent virulence tests. Genetically identical isolates were found within single oyster samples, but were not found between any experimentally treated shellfish. Ten isolates with unique PFGE profiles were randomly selected from each sample and tested for virulence (Table 21). Of isolates from fresh oysters, 20% were virulent. For both abuse temperatures, 40% of strains tested proved virulent. Chi-square demonstrated that no significant relationship existed between storage temperature and percentages of virulent isolates ($\chi^2 = 2.4$, $p=0.301$).

TABLE 21: Proportions of *Vibrio vulnificus* in oysters stored at elevated temperatures and associated virulence

Strain Designation	Virulence (+/-)	Strain Designation	Virulence (+/-)	Strain Designation	Virulence (+/-)
UN1a	-	20-1a	+	27-1a	+
UN2a	-	20-2a	-	27-2a	-
UN3a	-	20-3a	+	27-3a	+
UN4a	-	20-4a	+	27-4a	-
UN5a	-	20-5a	+	27-5a	-
UN6a	+	20-6a	+	27-6a	+
UN7a	-	20-7a	-	27-7a	-
UN8a	-	20-8a	-	27-8a	-
UN9a	+	20-9a	-	27-9a	-
UN10a	-	20-10a	-	27-10a	+
UN1b	-	20-1b	+	27-1b	+
UN2b	+	20-2b	-	27-2b	-
UN3b	-	20-3b	+	27-3b	-
UN4b	-	20-4b	+	27-4b	+
UN5b	-	20-5b	-	27-5b	-
UN6b	+	20-6b	-	27-6b	-
UN7b	-	20-7b	-	27-7b	+
UN8b	-	20-8b	-	27-8b	+
UN9b	-	20-9b	-	27-9b	-
20% virulent		40% virulent		40% virulent	

UN = fresh oysters

$p = 0.301$

20 = oysters stored at 20 degrees Celcius

27 = oysters stored at 27 degrees Celcius

mean concentration in fresh oysters = 1.6×10^6

mean concentration in oysters stored at 20 degrees Celcius = 1.4×10^4

mean concentration in oysters stored at 27 degrees Celcius = 9.0×10^3

The effects of thermal abuse on the concentration of virulent *V. vulnificus* isolates in shellfish may relate to their initial concentrations. In fresh oysters the initial proportion of virulent isolates was low. If a higher proportion of virulent isolates had been present in test samples, results may have differed. Due to an observed decrease in *V. vulnificus* levels in temperature abused oysters, results from this study are questioned when compared to other studies. Post-harvest temperature should continue to be kept low, since there is over-whelming evidence that low temperature correlates with low *V. vulnificus* levels in oysters (37, 149). However for reasons stated previously, low levels of virulent strains should not be used to assume lower disease risk. Interactions among genetically heterogeneous populations may be important for disease onset, so until new information is available, total levels of *V. vulnificus* should be monitored to assess human risk.

CONCLUSIONS

Results from this study have demonstrated the genetic and phenotypic diversity of *V. vulnificus*, as well as the complexity of accurate virulence assessment. Phenotypic characterization by FAME and biotype do not provide a definitive means for predicting virulence. Serological testing may eventually prove effective, but not until more antisera are available which identify higher percentages of environmental isolates. Furthermore, genetic profiling by PFGE and RT demonstrate such high diversity, that using these techniques may be to discriminatory. However, evidence that mixed populations of low and high virulence strains may initiate infections seems to undermine the urgency to differentiate strains by virulence. Knowing the degree of genetic diversity among *V. vulnificus* isolates, the disease process may operate as a "population." Understanding that populations of *V. vulnificus* are ingested, it may be important to consider the virulence of the entire population rather than the presence or absence of high virulent strains. Considering that key virulence factors such as capsule, extracellular enzyme secretion, and iron-binding ability are expressed in varying degrees by most environmental *V. vulnificus* strains, it may be of detriment to consumer safety to not consider that all strains are capable of initiating illness. Therefore, disease potential may be as much a characteristic of the population as it is of the individual. If disease manifestation depends on specific preexisting host-factors, as well as precise bacterial interactions within a population, this may explain the over-all low occurrence of *V.*

vulnificus illness among a large number of high-risk individuals. Even though the specific details of *V. vulnificus* virulence remains elusive, from these studies the following conclusions can be made:

1. Phenotypic characteristics (FAME, biotype, capsule-serotype, and LPS-serotype) were unable to differentiate *V. vulnificus* strains by virulence.
2. RFLP analysis by PFGE demonstrated great genetic diversity among *V. vulnificus* strains, and did not correlate with virulence.
3. RFLP analysis by RT demonstrated genetic diversity, but provided the best means for clustering *V. vulnificus* isolates.
4. RT analysis shows some association with geographical but not environmental source
5. Clinical isolates were not exclusive to a single RT, but were more prominent in clusters B and D than cluster C. Strains with low LD₅₀ values were found in both clusters B and C.
6. Virulent strains in case-associated oysters were present at approximately 10³ Vv/gram oyster meat.
7. The human infectious dose of *V. vulnificus* may be as low as 10³ Vv/ gram oyster meat.
8. Human blood specimens contained single virulent strains of *V. vulnificus*, ranging from 10⁷ to 10¹⁰/mL blood.
9. *Vibrio vulnificus* infections might be initiated by multiple genetically heterogeneous isolates with different LD₅₀ values.
10. Significantly higher percentages of virulent strains were collected from springtime oysters than from oysters in the summer or fall.
11. Post-harvest temperature abuse did not significantly increase the proportion virulent strains in shellfish.

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BIOGRAPHICAL SKETCH

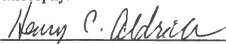
James Keith Jackson graduated from the University of Alabama at Birmingham in 1989 with a Bachelor of Science in biology. He continued his academic pursuits at Jacksonville State University, where he began working with shellfish and *Vibrio vulnificus*. While at Jacksonville State, he studied the effects of acute salinity change on *in vitro* phagocytosis of *V. vulnificus* by oyster immune cells (hemocytes). This research was important because hemocytes were believed to harbor viable *V. vulnificus* cells, believed to resist phagocytic elimination and intercellular enzyme degradation. In 1993 he received his Master of Science degree in biology and came to the University of Florida to pursue a doctorate. In his doctoral research, he has continued work with *V. vulnificus*, examining phenotypic and genotypic characteristics in an effort to differentiate high-virulent strains from those with low-virulence. He will graduate in August of 1997.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



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August 1997



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